PATENT Attorney Docket No. BA-32448(1)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	BAKER et al.
Serial No.	:	10/817,058
Filing Date	:	April 2, 2004
For	:	Method of Treating Cardiac Ischemia by Using Erythropoietin
Group Art Unit:		1653
Examiner	:	MAYER, Suzanne Marie
Confirmation No.:		2664

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 CFR §1.131

Sir:

I, John E. Baker, Ph.D., being an inventor and applicant in the above-identified patent application, declare and say as follows:

1. That on a date prior to December 29, 2000, I, Dr. John E. Baker, conceived of a method of increasing resistance of the heart to injury from ischemia utilizing erythropoietin (EPO). This is evidenced, at least in part, by the following exhibits:

(a) **Exhibit A**, which is a copy of a page of a research notebook dated *May 29, 1998*, with my observations and notes of a presentation on the identity of known triggers of the late phase of ischemic preconditioning. The notebook page includes my notation of the use of erythropoietin (EPO) to confer late preconditioning against injury from ischemia, where there is a time delay between administering erythropoietin and subjecting the heart to ischemia/reperfusion.

(b) **Exhibit B**, which is a copy of my notations on the backside of the confirmation of hotel reservation (dated March 30, 2000) prior to the International Symposium (The Developing Heart) in Prague, Czech Republic on May 18-20, 2000. The notations were made on a date between March 30, 2000 (receipt of hotel confirmation) and May 11, 2000 (date of travel to the Prague meeting). The notations were part of an outline in

preparation for my slide presentation for the Prague meeting, which was entitled "Chronic Hypoxia Increases Endothelial Constitutive Nitric Oxide Synthase and Decreases Caveolin-3". This document includes my notation of a proposed slide (not presented) that erythropoietin activates protein kinases, that chronic hypoxia induces production of erythropoietin, and that administering erythropoietin increases resistance to ischemia to confer early preconditioning (versus late preconditioning, Exhibit A).

(c) **Exhibit C**, entitled "Rationale", is a copy of a proposed slide that I prepared between March 30 to May 11, 2000, for my talk at the Prague meeting (May 18-20, 2000), but was not included in the presentation. The slide records my rationale for administering erythropoietin prior to an ischemic event (and in the absence of chronic hypoxia) to result in an increased level of erythropoietin which will activate protein kinases which will increase resistance to myocardial ischemia and confer early preconditioning for an immediate cardioprotective effect from ischemia.

(d) **Exhibit D** is a copy of a slide that I prepared between March 30 to May 11, 2000, for my talk at the Prague meeting (May 18-20, 2000), but was not included in the presentation. The slide records the experimental conditions to conduct an animal study to demonstrate immediate cardioprotection by erythropoietin. The slide presents the experimental protocol of

-- administering an amount of erythropoietin of 0-100 U/ml – to achieve that concentration in the blood

-- a single treatment for an about 15 minute period prior to the ischemic event ("perfusion plus drug")

-- to activate protein kinases and nitric oxide synthase (NOS) prior to the ischemic event

-- to result in resistance to ischemia

(e) **Exhibit E** is a copy of a document that I prepared on or about *August 10, 2000*, which records the experimental conditions to conduct an animal study to demonstrate immediate cardioprotection by administering erythropoietin when given prior to an ischemic event, during an ischemic event, and after an ischemic event.

2. That from the date of conception *prior to December 29, 2000 to April 4, 2003*, I, Dr. John E. Baker, in part with Dr. Yang Shi, diligently pursued this invention up to the April 4, 2003 date of filing the provisional application S/N 60/460,684 to the above-identified patent application in the U.S. Patent and Trademark Office.

(a) I conducted initial research studies *from May 2000 to December 2001* to determine the mechanisms stimulated by chronic hypoxia that result in increased resistance to ischemia.

(i) These studies were conducted in order to develop a model to use in testing and validating the effect of administering erythropoietin to cause the same effect as chronic hypoxia (i.e., increased protection of the heart against injury from ischemia) but in the absence of chronic hypoxia.

(ii) As I had set forth in **Exhibits C and D**, my rationale was that administering erythropoietin prior to an ischemic event (and in the absence of chronic hypoxia) would result in an increased level of erythropoietin to activate protein kinases and nitric oxide synthase which will result in resistance to ischemia.

(iii) The results of these research studies showed that increased resistance to ischemia in a chronic hypoxia situation is due, at least in part, to the activity of potassium channels and generation of nitric oxide synthase.

(b) I published the results of these initial research studies that describe my research relating to the mechanisms stimulated by chronic hypoxia that result in increased resistance to ischemia in the following publications.

Exhibit F: Eells et al., "Increased Mitochondrial K_{ATP} Channel Activity During Chronic Myocardial Hypoxia," *Circulation Research* 87: 915-921 (2000), reporting study data showing mitochondrial K_{ATP} channels mediate cardioprotection in chronically hypoxic hearts. This study was conducted between March 1998 to July 2000.

Exhibit G: Kong et al., "Sarcolemmal and Mitochondrial K_{ATP} Channels Mediate Cardioprotection in Chronically Hypoxic Hearts," *Journal of Molecular and Cellular Cardiology* 33: 1041-1045 (2001), reporting study data showing that both sarcolemmal and mitochondrial K_{ATP} channels contribute to cardioprotection in chronically hypoxic hearts. This study was conducted between January to December 2000.

Exhibit H: Shi et al., "Chronic Hypoxia Increases Endothelial Nitric Oxide Synthase Generation of Nitric Oxide by Increasing Heat Shock Protein 90 Association and Serine Phosphorylation," *Circulation Research* 91: 300-306 (2002), reporting study data relating to a role for nitric oxide in protecting chronically hypoxic hearts against injury from ischemia. This study was conducted between December 2000 to January 2002.

(c) Based on the results of the studies, I developed a model that involved monitoring the activity of potassium channels and the level of nitric oxide to test and validate the effects of EPO to cause the same effect as chronic hypoxia (i.e., increased protection of the heart against injury from ischemia) and confer immediate cardioprotection in the absence of chronic hypoxia.

(d) On or about *September 11, 2001*, I submitted a research proposal entitled "Erythropoietin, Nitric Oxide Synthase and Resistance to Myocardial Ischemia" (a copy of which is attached as **Exhibit I**) to test whether EPO increases nitric oxide production in a normoxic animal model to confer resistance to ischemia in the absence of chronic hypoxia. This research proposal was based, at least in part, on my prior research (**Exhibit H**) on rabbits adapted to chronic hypoxia, and showed that increased resistance to ischemia in a chronic hypoxia situation is due to the generation of nitric oxide synthase.

(e) On or about *December 19, 2001*, Dr. Yang Shi and I (Dr. John E. Baker) directed and supervised a research study on administering erythropoietin to demonstrate and confirm the effect of administering EPO as an early preconditioning treatment to

increase circulating EPO levels in the absence of chronic hypoxia to increase resistance to ischemia caused by an ischemic event.

(f) On or about *May 9, 2002*, Dr. Yang Shi and I (Dr. John E. Baker) submitted a Discovery Record and Report entitled "Cardioprotection by Erythropoietin" to the Medical College of Wisconsin (MCW) Research Foundation, a copy of which is attached as **Exhibit J.** Paragraphs 4 and 5d evidence the results of our research study conducted on or about December 19, 2001.

(i) As set forth in the attached "Brief description of the discovery," in the study, hearts isolated from rabbits were perfused with a range of concentrations of erythropoietin prior to a global ischemic insult followed by reperfusion, and the results showed cardioprotection by the administration of EPO.

(ii) The results were based on the previously developed model involving monitoring the activity of potassium channels and the level of nitric oxide.

(g) *From May 2002 to April 2003*, I conducted additional research studies to establish the role of protein kinases to protect the heart against ischemic injury under conditions of chronic hypoxia.

(i) These studies were conducted to develop a model involving monitoring the level of protein kinase to use in testing and validating the effect of administering erythropoietin in the absence of chronic hypoxia conditions to cause the same effect as chronic hypoxia to activate protein kinase levels to increase resistance to ischemia.

(ii) The results of these studies showed that increased resistance to ischemia in a chronic hypoxia situation is due, at least in part, to the activation of protein kinases.

(h) I published the results of the studies conducted *between December 2001 and April 2003* in the following publications.

Exhibit K: Rafiee et al., "Activation of Protein Kinases in Chronically Hypoxic Infant Human and Rabbit Hearts: Role in Cardioprotection," *Circulation* 106: 239-245 (2002), reporting study data on infant human and rabbit hearts adapted to chronic hypoxia through activation of protein kinases and pathways responsible for protecting the chronically hypoxic heart against injury from ischemia-reperfusion.

Exhibit L: Rafiee et al., "Cellular Redistribution of Inducible Hsp70 Protein in the Human and Rabbit Heart in Response to the Stress of Chronic Hypoxia: Role of Protein Kinases," *Journal of Biological Chemistry* 278: 43636-43644 (*2003*), reporting study data showing the expression and distribution of heat shock proteins in chronically hypoxic hearts are influenced by several protein kinases.

Exhibit M: Shi et al., "Acute cardioprotective effects of erythropoietin in infant rabbits are mediated by activation of protein kinases and potassium channels," *Basic Res. in Cardiol.* 99: 173-182 (2004), reporting the data from our research on the action of EPO on activation of protein kinase signaling pathways and potassium channels to confer cardioprotective effects in the absence of chronic hypoxia. This data was initially submitted as part of the disclosure in the provisional application S/N 60/460,684 (filed *April 4, 2003*)

(i) At a time *between May 9, 2002 and April 4, 2003*, I met with Dr. Joseph Hill, the Acting Director of the MCW Research Foundation to discuss the filing of a provisional patent application, and provided him with additional details regarding the invention disclosure for the purpose of preparing the provisional application.

4. That on *April 4, 2003*, the provisional application S/N 60/460,684 to the above-identified patent application, entitled "Method of Treating Cardiac Ischemia by Using Erythropoietin," was filed in the U.S. Patent and Trademark Office.

5. I further hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

Date: March 15, 2007 By: John E. Baker

John E. Baker, Ph.D.

Measure mRNA, activity Nox/3 cGMP 5/29/98

Bolli

role of NO in Late preconditioning

ISCHEMIC STRESS — Nonspecific NO, No ROS

Trigger of Late PC

Signal transduction cascade — PKC, TyrK

gene upregulation

MEDIATOR OF LATE PC — Antioxidants, HSPs, NO

PROTECTION

2-4

protective effects of Late PC
mediated by NO in absence of ischemia
Takano/Bolli
Circ Rs 1998

ANO
IAP
st
ster
tes

contractile function?

Would erythropoietin confer Late preconditioning? (3)

Which NOS is activated

Amino guanidine – i NOS
SMT – i NOS

H_1: Late preconditioning not possible in iNOS deficient mice

Exhibit A

Exhibit B

THE DEVELOPING HEART
PRAGUE, CZECH REPUBLIC
MAY, 18-20, 2000

CONFIRMATION OF HOTEL RESERVATION
FOR HONORARY GUESTS
International Symposium
THE DEVELOPING HEART
Prague, Czech Republic, May 18-20, 2000

DATE: **Prague, March 30, 2000**

NAME: **Prof. Dr. J. E. BAKER**

HOTEL: **Vila Lanna** - guest-house of the Czech Academy of Sciences
V Sadech 1, 160 00 Prague 6,
tel.: 02- 2432 1278
fax: 02- 2432 0316
The Vila Lanna is located in quiet surroundings, less than 10 minutes walking from underground line A station Hradčanská.

ROOM: 1 single room with breakfast

DATE of stay: **May 17 - 21 (four nights)**
If the dates are not correct please let us know by return

ACCOMPANYING PERSONS: 0

Connection to DIPLOMAT: by underground line A from station Hradčanská to station Dejvická (one stop, about 5 minutes) or about 15 minutes walking

Connection to CHARLES UNIVERSITY (Get-together party) : by underground line A from station Hradčanská to station Můstek (three stops, about 10 minutes)

TOURIST PROGRAMME : not required

TO BE PAID: **hotel accommodation covered by organizing commitee**

We offer you transportation from the airport to your hotel; for this case we would need the **precise date of your arrival and departure (flight no).**
If you must change or cancel your reservation, please write us immediately.
If you have any questions, please do not hesitate to contact CBT Travel Agency.

Looking forward to hearing from you soon,

Yours sincerely,

Zina Pechlivanidis
CBT Travel Agency Ltd., Staroměstské nám. 17, Prague 1, Czech Republic
Fax: 420-2 24 22 47 24, Tel.: 420- 2 24 22 46 46, e-mail cbttravl@mbox.vol.cz

Exhibit B
continued

SLIDES

Chronic hypoxia → increased resistance to ischemia

Clinical + basic science background

Mechanisms to explain phenotypic changes

nitric oxide < mRNA (-NO donor)
protein (NOS inhibitor)

caveolin-3 mRNA NOS 1, 2, 3
IP NOS3: IB Cav-3
Cav-3 protein
$NO_2^- + NO_3^-$ Levels

erythropoietin
- activates protein kinases
- activates NOS (isoforms)
- chronic hypoxia induces erythropoiesis.
- candidate to increase resistance to ischemia.

protein kinases – JNK, p38, PKC(ε)

KATP channels – sarcolemmal + mito

Acknowledge Gecks started all of this in 1958!

RATIONALE

CHRONIC HYPOXIA → ACTIVATION OF PKC, p38 MAP KINASE, JUN KINASE → ↑ RESISTANCE TO MYOCARDIAL ISCHEMIA

CHRONIC HYPOXIA → ↑ EPO → ERYTHROPOIESIS

EPO → ACTIVATION OF PKC, p38 MAP KINASE, JUN KINASE

Hypothesis:

EPO → ACTIVATION OF PROTEIN KINASES → ↑ RESISTANCE TO MYOCARDIAL ISCHEMIA

Exhibit C

Infant NZW rabbit
Isolated heart model
Aerobic perfusion with bicarbonate buffer

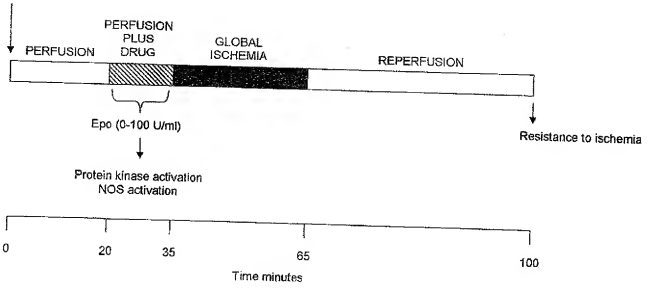
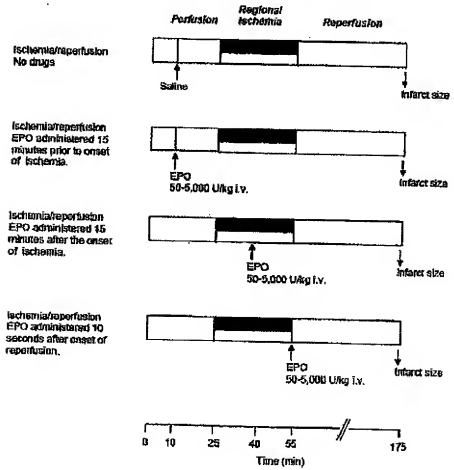


Exhibit D

Exhibit E

ERYTHROPOIETIN LIMITS MYOCARDIAL INJURY FOLLOWING ISCHEMIA/REPERFUSION: PHASE OF ACTION

8 week old male Sprague Dawley rat
In vivo model of regional myocardial ischemia/reperfusion
N = 6/group



John E. Baker, Ph.D.
August 10, 2000

Exhibit F

Increased Mitochondrial K_{ATP} Channel Activity During Chronic Myocardial Hypoxia

Is Cardioprotection Mediated by Improved Bioenergetics?

Janis T. Eells, Michele M. Henry, Garrett J. Gross, John E. Baker

Abstract—Increased resistance to myocardial ischemia in chronically hypoxic immature rabbit hearts is associated with activation of ATP-sensitive K^+ (K_{ATP}) channels. We determined whether chronic hypoxia from birth alters the function of the mitochondrial K_{ATP} channel. The K_{ATP} channel opener bimakalim (1 μmol/L) increased postischemic recovery of left ventricular developed pressure in isolated normoxic (Fio_2=0.21) hearts to values (42±4% to 67±5%) not different from those of hypoxic controls but did not alter postischemic recovery of developed pressure in isolated chronically hypoxic (Fio_2=0.12) hearts (69±5% to 72±5%). Conversely, the K_{ATP} channel blockers glibenclamide (1 μmol/L) and 5-hydroxydecanoate (5-HD, 300 μmol/L) attenuated the cardioprotective effect of hypoxia but had no effect on postischemic recovery of function in normoxic hearts. ATP synthesis rates in hypoxic heart mitochondria (3.92±0.23 μmol ATP · min^{-1} · mg mitochondrial $protein^{-1}$) were significantly greater than rates in normoxic hearts (2.95±0.08 μmol ATP · min^{-1} · mg mitochondrial $protein^{-1}$). Bimakalim (1 μmol/L) decreased the rate of ATP synthesis in normoxic heart mitochondria consistent with mitochondrial K_{ATP} channel activation and mitochondrial depolarization. The effect of bimakalim on ATP synthesis was antagonized by the K_{ATP} channel blockers glibenclamide (1 μmol/L) and 5-HD (300 μmol/L) in normoxic heart mitochondria, whereas glibenclamide and 5-HD alone had no effect. In hypoxic heart mitochondria, the rate of ATP synthesis was not affected by bimakalim but was attenuated by glibenclamide and 5-HD. We conclude that mitochondrial K_{ATP} channels are activated in chronically hypoxic rabbit hearts and implicate activation of this channel in the improved mitochondrial bioenergetics and cardioprotection observed. (*Circ Res.* 2000;87:915-921.)

Key Words: chronic hypoxia ■ 5-hydroxydecanoate ■ mitochondrial K_{ATP} channel

The ATP-sensitive K^+ channel (K_{ATP} channel) is an important mediator of cellular protection in response to myocardial oxygen deprivation after chronic hypoxia and ischemia. Adaptation of hearts to chronic hypoxia results in enhanced activation of K_{ATP} channels.[1] Increased resistance to ischemia exhibited by chronically hypoxic rabbit hearts is associated with increased activation of the K_{ATP} channel.[2] Preconditioning in normoxic immature rabbit hearts is also associated with activation of the K_{ATP} channel.[3]

The precise cellular location at which the K_{ATP} channel mediates cardioprotection is unknown. If this can be identified, then the mechanisms through which K_{ATP} channels exert their protective effect may be determined. The cardioprotective effect of K_{ATP} channel openers, used at concentrations that do not shorten action potential duration, are abolished by the K_{ATP} channel blocker 5-hydroxydecanoate (5-HD).[4] Thus, 5-HD does not appear to act on the sarcolemmal K_{ATP} channel. K_{ATP} channels are also found in the inner mitochondrial membrane[5,6] where they control mitochondrial volume.[7,8] However, it is unknown if this K_{ATP} channel is involved in mitochondrial energy production.[9] Diazoxide, a K_{ATP} channel opener, is 1000 times more selective for opening mitochondrial K_{ATP} channels than sarcolemmal channels.[7] The cardioprotective effect of diazoxide during ischemia is abolished by 5-HD, suggesting a role for the mitochondrial K_{ATP} channel in protection of the ischemic myocardium.[10] 5-HD abolished the cardioprotective effects of preconditioning in immature hearts, suggesting a cardioprotective role for mitochondrial K_{ATP} channels in immature hearts during conditions of oxygen deprivation.[3]

The present study further explores the involvement of mitochondria in the adaptation of heart muscle to chronic hypoxia. We hypothesize that activation of the mitochondrial K_{ATP} channel and its impact on mitochondrial bioenergetics may be an important event associated with increased resistance to ischemia in hearts adapted to chronic hypoxia. To assess the contribution of mitochondrial K_{ATP} channels, the rate of mitochondrial ATP synthesis was compared in normoxic and chronically hypoxic hearts. Our findings indicate that acute activation of the mitochondrial K_{ATP} channel

Received July 26, 2000; revision received September 19, 2000; accepted September 19, 2000.
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Correspondence to Janis T. Eells, PhD, Department of Pharmacology and Toxicology, Medical College of Wisconsin, 8701 Watertown Plank Rd, Milwaukee, WI 53226. E-mail jeells@mcw.edu

Circulation Research is available at http://www.circresaha.org

TABLE 1. Hemodynamic Values for Each Group

Groups	Before Drug			After Drug			Reperfusion (35 Minutes)			
	Heart Rate, bpm	Coronary Flow Rate, mL/min	LVDP, mm Hg	Heart Rate, bpm	Coronary Flow Rate, mL/min	LVDP, mm Hg	Heart Rate, bpm	Coronary Flow Rate, mL/min	LVDP, mm Hg	Percent Recovery LVDP
Normoxic, no intervention, control for bimakalim and glibenclamide	232±29	6±1	99±8	...	...	...	222±34	5±1	42±6†	42±4
Normoxic+bimakalim (1 μmol/L)	231±19	6±1	99±6	270±16	12±1	96±5	228±17	5±2	66±7†	67±5
Normoxic+glibenclamide (1 μmol/L)	229±16	6±1	98±6	168±21*	3±2*	52±6*	221±28	6±1	42±8†	43±5
Normoxic, no intervention, control for 5-HD	225±28	6±2	102±7	...	...	...	210±28	5±1	45±4†	44±4
Normoxic+5-HD (300 μmol/L)	240±16	6±2	97±6	225±23	6±2	95±6	225±28	6±2	40±6†	41±4
Hypoxic, no intervention, control for bimakalim and glibenclamide	224±21	8±1‡	100±6	...	...	...	222±28	7±1	69±8†	69±5
Hypoxic+bimakalim (1 μmol/L)	230±19	8±1‡	96±9	269±18	14±3	76±6*	219±16	5±2	69±10†	72±5
Hypoxic+glibenclamide (1 μmol/L)	230±19	8±2‡	92±9	162±20*	4±2*	48±12*	206±31	7±2	40±9†	43±4
Hypoxic, no intervention, control for 5-HD	221±16	9±2‡	100±6	...	...	...	210±23	8±1	67±4†	67±5
Hypoxic+5-HD (300 μmol/L)	236±11	10±2‡	102±6	210±23	9±2	103±7	210±23	7±2	53±5†	52±5

LVDP indicates left ventricular developed pressure. Values are mean±SD from 6 hearts per group.
*$P<0.05$ before drug vs after drug; †$P<0.05$ before drug vs reperfusion; and ‡$P<0.05$ normoxic vs hypoxic.

increases K^+ influx into mitochondria, resulting in a reduction in the driving force for ATP synthesis. In addition, these findings indicate that K_{ATP} channels are tonically active in mitochondria isolated from hypoxic hearts and that this tonic activity may play a role in the alteration of mitochondrial bioenergetics, which renders the hypoxic heart more resistant to myocardial ischemia.

Materials and Methods

Creation of Hypoxia From Birth

Pregnant New Zealand White rabbits were obtained from New Franken Research Rabbits (New Franken, Wis). Animals used in this study received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals*, formulated by the National Research Council, 1996. For the hypoxic studies, the kits were born in a normoxic environment and then transferred to a hypoxic environment (Fio_2=0.12) immediately after their first feeding.[1-3] The oxygen in the chamber was maintained at this level throughout the remainder of the study. For normoxic studies, the kits were raised under identical conditions except that Fio_2 in the environmental chamber remained at 0.21 for the duration of the study. The age of the rabbits at the time of the study was 7 to 10 days.

Assessment of Ventricular Function

The isolated rabbit heart model was used for these studies and was instrumented as previously described.[3,11] The standard perfusate used was Krebs-Henseleit bicarbonate buffer.[12] Immediately after aortic cannulation, hearts were perfused at a constant pressure of 43 mm Hg in the Langendorff mode for 30 minutes, during which time balloons were placed in both the left and right ventricles. Biventricular function and coronary flow rate were then recorded under steady-state conditions.[2,3] Hearts were then perfused with either a K_{ATP} opener (bimakalim, 1 μmol/L) or a K_{ATP} blocker (glibenclamide, 1 μmol/L, or 5-HD, 300 μmol/L) for another 15 minutes before a 30-minute period of global, no-flow ischemia at 39°C. After the ischemic period, hearts were reperfused for 35 minutes, during which time the various indexes of cardiac function were again measured under steady-state conditions. Thus, each heart served as its own control.

Mitochondrial ATP Synthesis, Membrane Potential, and Ventricular ATP Concentrations

Mitochondria were isolated from normoxic and hypoxic hearts by differential centrifugation as described by Solem and Wallace.[13] Cardiac mitochondria prepared by this methodology have been shown to be metabolically active with respiratory control ratios of 3.5 to 5.0 with succinate and 8.0 to 10.0 with glutamate/malate and corresponding ADP/O_2 ratios of 1.5 to 1.7 and 2.5 to 2.7. Mitochondrial ATP synthesis was measured in the presence of complex I substrates (pyruvate plus malate) as previously described.[14] Semiquantitative measurements of the potential difference across the inner mitochondrial membrane were determined spectrofluorometrically using the dye rhodamine-123.[15] ATP concentrations were determined in ventricular tissue extracts by luciferin-luciferase luminometry.[14,16,17]

Statistical Analysis

Recovery of developed pressure was expressed as a percentage of its predrug value. A minimum of 6 hearts was used for each of the 10 conditions studied, and the results are expressed as mean±SD or

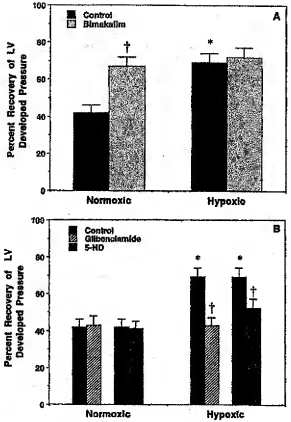

Figure 1. Effect of K_{ATP} channel openers and blockers on the postischemic recovery of ventricular function in normoxic and chronically hypoxic immature rabbit hearts. The K_{ATP} channel opener bimakalim (1 μmol/L) (**A**) or the K_{ATP} channel blockers glibenclamide (1 μmol/L) or 5-HD (300 μmol/L) (**B**) were added 15 minutes before a global ischemic period of 30 minutes, followed by 35 minutes of reperfusion. Results are expressed as percent recovery of left ventricular pressure. Data shown are the mean±SE from 6 experiments. *P<0.05 normoxic vs hypoxic; †P<0.05 control vs drug-treated. LV indicates left ventricular.

mean±SE. Statistical analysis was performed by use of repeated-measures ANOVA, with the Greenhouse-Geisser adjustment used to correct for the inflated risk of a type I error.[18] After ANOVA, the data were corrected for multiple comparisons. Significance was accepted at a level of P<0.05.

Results

Contribution of the K_{ATP} Channel to Postischemic Recovery of Ventricular Function in Normoxic and Chronically Hypoxic Immature Rabbit Hearts

Table 1 and Figure 1 illustrate the effects of bimakalim (1 μmol/L), glibenclamide (1 μmol/L), and 5-HD (300 μmol/L) on the recovery of postischemic left ventricular function in hearts from normoxic and hypoxic rabbits perfused at constant pressure. These experiments were conducted using the same concentrations of K_{ATP} channel openers and blockers in the perfused hearts used to examine K_{ATP} function in isolated mitochondria. Recovery of postischemic left ventricular developed pressure in normoxic and hypoxic hearts was 42±4% (45±4% for the normoxic no-intervention control for 5-HD) and 69±5% (67±4% for the hypoxic no-intervention control for 5-HD), respectively, consistent with our previous findings showing that hypoxia increases the tolerance of the heart to subsequent ischemia.[2] As shown in Figure 1A, the K_{ATP} channel opener bimakalim (1 μmol/L) increased recovery in normoxic hearts from 42±4% to 67±5% but had no effect on recovery of function in hypoxic hearts (72±5%). Thus, bimakalim increased the recovery of normoxic hearts to that observed in hypoxic hearts but did not alter functional recovery in hypoxic hearts. Conversely, the K_{ATP} channel blockers glibenclamide (1 μmol/L) and 5-HD (300 μmol/L) had no effect on recovery of developed pressure in normoxic hearts but decreased recovery in hypoxic hearts from 69±5% to 43±4% in experiments conducted with glibenclamide (1 μmol/L) and from 67±5% to 52±5% in experiments conducted with 5-HD (300 μmol/L) (Figure 1B).

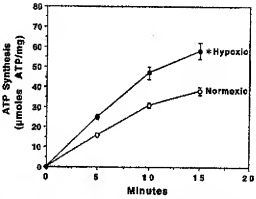

Figure 2. ATP synthesis in intact mitochondria isolated from hearts of normoxic and chronically hypoxic immature rabbits. ATP synthesis was measured in the presence of complex I substrates (1 mmol/L pyruvate+1 mmol/L malate) in mitochondria isolated from normoxic and hypoxic hearts. Results are expressed as μmol ATP · min^{-1} · mg mitochondrial $protein^{-1}$. Data shown are the mean±SE from 4 to 6 experiments. *P<0.05 normoxic vs hypoxic.

Effect of Chronic Hypoxia on Mitochondrial ATP Synthesis and Myocardial Energy Metabolism

ATP synthesis was measured in mitochondria isolated from hearts of normoxic and chronically hypoxic rabbits in the presence of the complex I substrates pyruvate and malate.[14,16,17] As shown in Figure 2, the rate of ATP synthesis in cardiac mitochondria was linear for 10 to 12 minutes in mitochondria isolated from both normoxic and hypoxic rabbits. The rate of ATP production in hypoxic heart mitochondria (3.82±0.23 μmol ATP · min^{-1} · mg mitochondrial $protein^{-1}$) was significantly greater than the rate of ATP production in normoxic heart mitochondria (2.95±0.08 μmol ATP · min^{-1} · mg mitochondrial $protein^{-1}$). ATP concentrations before the addition of respiratory substrates were 63±4 μmol/mg of mitochondrial protein in normoxic heart mitochondria and 73±12 μmol/mg in hypoxic heart mitochondria.

Other differences in myocardial energy metabolism were also apparent in chronically hypoxic versus normoxic immature rabbit hearts. Table 2 shows that ventricular lactate concentrations were twice as high in hypoxic hearts than normoxic hearts and ventricular lactate dehydrogenase

TABLE 2. Ventricular Energy Metabolites

	Normoxic (Fio_2=0.21)	Hypoxic (Fio_2=0.12)
Ventricular lactate,* μmol/g dry wt	2±1	4±1†
Ventricular LDH,* IU/g wet wt	450±51	608±59†
Ventricular ATP, nmol ATP/mg tissue protein	9.63±1.32	12.14±1.42
Rate of mitochondrial ATP synthesis, μmol ATP · min^{-1} · mg mitochondrial $protein^{-1}$	2.95±0.08	3.82±0.23†

Values are mean±SD from a minimum of 6 hearts in each group.
*Data are from Baker et al,[2] 1997.
†$P<0.05$ normoxic vs hypoxic.

(LDH) concentrations were 35% greater in hypoxic than normoxic hearts. In addition, we have previously reported a shift in the LDH isoform distribution toward the M or LD5 isoform in hypoxic hearts.[2] These changes are indicative of an increased dependency on anaerobic glycolysis for energy production in hypoxic hearts. The combination of increased mitochondrial ATP production and increased glycolytic ATP production is likely to be responsible for the observation that myocardial ATP concentrations did not differ between normoxic and hypoxic hearts (Table 2).

K_{ATP} Channel–Mediated Alterations in Mitochondrial ATP Synthesis

Activation of the mitochondrial K_{ATP} channel has been shown to increase K^+ influx into the mitochondrial matrix, resulting in mitochondrial membrane depolarization and a reduction in the driving force for ATP synthesis.[5,8,10] The effects of K_{ATP} channel openers and blockers on ATP synthesis in mitochondria isolated from normoxic rabbit hearts are shown in Figures 3 and 4. As shown in Figure 3, the K_{ATP} channel opener bimakalim inhibited the rate of ATP synthesis in mitochondria isolated from normoxic rabbit hearts. In the presence of 1 μmol/L bimakalim, the rate of ATP synthesis was reduced from 2.96±0.10 μmol ATP · min^{-1} · mg mitochondrial $protein^{-1}$ to 1.56±0.22 μmol ATP · min^{-1} · mg mitochondrial $protein^{-1}$, a 52% reduction in the rate of ATP synthesis. The inhibitory action of bimakalim on mitochondrial ATP synthesis was sensitive to the K_{ATP} channel blocker glibenclamide (1 μmol/L). Glibenclamide (1 μmol/L) alone had no effect on the rate of ATP synthesis in normoxic heart mitochondria. However, the addition of glibenclamide (1 μmol/L) before the addition of bimakalim prevented the inhibition of ATP synthesis mediated by bimakalim. Figure 3 also shows that the reduction in ATP synthesis mediated by bimakalim (1 μmol/L) was abolished by the mitochondrial selective K_{ATP} blocker 5-HD (300 μmol/L). As with glibenclamide, 5-HD alone had no effect on the rate of mitochondrial ATP synthesis but prevented the reduction of ATP synthesis mediated by bimakalim.

Data presented in Figure 4 show that the mitochondria-specific K_{ATP} channel opener diazoxide (100 μmol/L) also reduced the rate of ATP synthesis from 3.04±0.30 μmol ATP · min^{-1} · mg mitochondrial $protein^{-1}$ to 2.03±0.30 μmol ATP · min^{-1} · mg mitochondrial $protein^{-1}$, a 32% reduction in the rate of ATP synthesis. Furthermore, in nominally

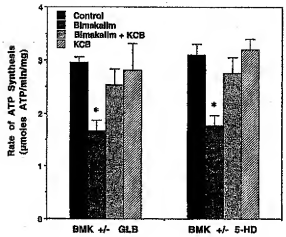

Figure 3. Effect of K_{ATP} channel openers and blockers on mitochondrial ATP synthesis in mitochondria isolated from normoxic immature rabbit hearts. Mitochondria isolated from normoxic hearts were incubated in the presence of vehicle (Control); bimakalim (1 μmol/L); bimakalim (1 μmol/L)+glibenclamide (1 μmol/L); glibenclamide (1 μmol/L) alone; bimakalim (1 μmol/L)+5-HD (300 μmol/L); or 5-HD (300 μmol/L) alone, and ATP synthesis was measured. Bimakalim inhibited ATP synthesis. The effect of bimakalim was antagonized by both glibenclamide and 5-HD, and glibenclamide or 5-HD alone had no effect on the rate of ATP synthesis. Results are expressed as μmol ATP · min^{-1} · mg mitochondrial $protein^{-1}$. Data shown are the mean±SE from 4 to 6 experiments. *$P<0.05$ control vs drug-treated. KCB indicates K_{ATP} channel blocker; GLB, glibenclamide; and BMK, bimakalim.

K^+-free medium, diazoxide (100 μmol/L) had no effect on the rate of mitochondrial ATP synthesis indicating that the effect of K_{ATP} channel openers on mitochondrial ATP synthesis is dependent on the electrochemical gradient for K^+. The reduced rates of mitochondrial ATP synthesis measured in nominally K^+-free medium are likely due to an increase in K^+-H^+ antiport activity.[7,8] Although it is possible that a reduction in ATP synthesis might interfere with the action of K_{ATP} channel openers, the similarity of our findings with other studies demonstrating that the effects of K_{ATP} channel openers on mitochondrial membrane potential and mitochondrial swelling are dependent on the electrochemical gradient for K^+ support this interpretation.[8,9]

Effects of K_{ATP} Channel Openers and Blockers on ATP Synthesis in Mitochondria Isolated From Normoxic and Chronically Hypoxic Hearts

Figure 5A compares the effect of bimakalim on mitochondrial ATP synthesis in normoxic and hypoxic heart mitochondria. In mitochondria isolated from normoxic hearts, bimakalim produced a concentration-dependent decrease in the rate of ATP synthesis, reducing the rate of synthesis 50% at 1 μmol/L and 60% at 10 μmol/L. The rate of ATP synthesis in hypoxic heart mitochondria was not affected by the K_{ATP} channel opener bimakalim at concentrations of 1 or 10 μmol/L. Figure 5B compares the effect of the K_{ATP} blockers glibenclamide (1 μmol/L) and 5-HD (300 μmol/L) on mitochondrial ATP synthesis in mitochondria isolated from normoxic and chronically hypoxic immature rabbit hearts. Nei-

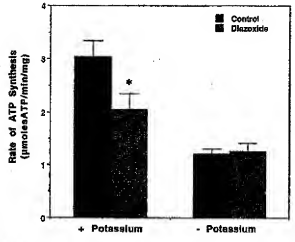

Figure 4. K^+ dependence of the effect of diazoxide on ATP synthesis in mitochondria isolated from normoxic immature rabbit hearts. ATP synthesis was measured in the presence of vehicle (Control) or diazoxide (100 μmol/L) in normoxic heart mitochondria incubated in buffer containing 110 mmol/L K^+ and in nominally K^+-free solution (KCl replaced with 110 mmol/L choline chloride, K_2HPO_4 replaced with 5 mmol/L Na_2HPO_4, and pH adjusted with trizma base). Diazoxide (100 μmol/L) produced a K^+-dependent inhibition of ATP synthesis in mitochondria isolated from normoxic hearts. Data shown are the mean±SE from 4 experiments. *P<0.05 control vs drug-treated.

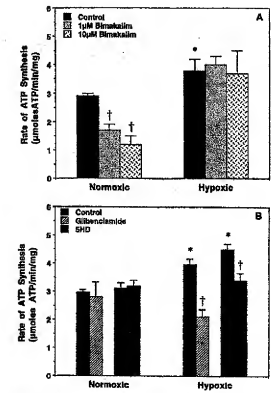

Figure 5. Effect of K_{ATP} channel openers and blockers on ATP synthesis in mitochondria isolated from normoxic and chronically hypoxic immature rabbit hearts. A, ATP synthesis was measured in normoxic heart mitochondria incubated in the presence of vehicle (Control) or bimakalim at concentrations 1 or 10 μmol/L. Bimakalim produced a concentration-dependent inhibition of ATP synthesis in mitochondria isolated from normoxic hearts; however, bimakalim had no effect on the rate of ATP synthesis in mitochondria isolated from chronically hypoxic hearts. B, ATP synthesis was measured in normoxic or hypoxic heart mitochondria treated with glibenclamide (1 μmol/L) or 5-HD (300 μmol/L). Glibenclamide and 5-HD had no effect on the rate of ATP synthesis in normoxic heart mitochondria. In contrast, both K_{ATP} channel blockers inhibited the rate of ATP synthesis in hypoxic heart mitochondria. Data shown are the mean±SE from 4 experiments. *P<0.05, normoxic vs hypoxic; †P<0.05 control vs drug-treated.

ther K_{ATP} blocker altered the rate of ATP synthesis in normoxic heart mitochondria; however, in hypoxic heart mitochondria, both glibenclamide and 5-HD significantly reduced the rate of ATP synthesis. Glibenclamide produced a 50% decrease in the rate of ATP synthesis and 5-HD reduced ATP synthesis by 25%.

Mitochondrial Membrane Potential in Mitochondria Isolated From Normoxic and Chronically Hypoxic Hearts

Semiquantitative measurements of mitochondrial membrane potential were determined using the fluorescent probe rhodamine-123.[15] In the absence of K_{ATP} channel modulators, resting membrane potential was remarkably similar in mitochondria isolated from normoxic and hypoxic hearts. Isolated cardiac mitochondria have been reported to have a membrane potential of −180±15 mV in studies using the potential sensitive probe tetraphenylphosphonium.[9] Attempts to assess the effects of K_{ATP} channel openers or blockers in mitochondria isolated from normoxic and hypoxic hearts using rhodamine-123 were confounded by interactions between the vehicle or the drugs and the fluorescent probe.

Discussion

We have demonstrated in rabbits that chronic exposure to hypoxia from birth increases the resistance of the heart to subsequent ischemia[1,2,11] and that glibenclamide, a K_{ATP} channel blocker, abolishes this cardioprotective effect. More recently, we have shown that ischemic preconditioning in immature rabbit hearts also increased resistance to ischemia and that 5-HD abolished this cardioprotective effect.[3] Thus, ischemic preconditioning and adaptation to chronic hypoxia in immature hearts appear to share a final common effector, the K_{ATP} channel.

In light of recent studies implicating the mitochondrial K_{ATP} channel in cardioprotection,[10,18–24] we conducted experiments to examine the role of the mitochondrial K_{ATP} channel in adaptation to chronic hypoxia in immature hearts. To assess mitochondrial K_{ATP} channel function, we measured the effect of several K_{ATP} channel openers and blockers on mitochondrial ATP synthesis in metabolically active mitochondria isolated from hearts of normoxic and chronically hypoxic rabbits. This approach was predicated on the knowledge that activation of the mitochondrial K_{ATP} channel has been shown to increase the influx of K^+ into mitochondria, resulting in mitochondrial depolarization and a reduction in the rate of ATP synthesis.[9] The actions of K_{ATP} channel openers and blockers in mitochondria isolated from

normoxic and hypoxic hearts paralleled their actions on cardiac function in isolated perfused hearts. K_{ATP} channel activation by bimakalim resulted in a decrease in the rate of ATP synthesis in normoxic heart mitochondria but had no effect on ATP synthesis in hypoxic heart mitochondria. Similarly, K_{ATP} channel activation markedly enhanced recovery of ventricular function in normoxic hearts but had no effect on functional recovery in hypoxic hearts. In normoxic heart mitochondria, the K_{ATP} blockers glibenclamide and 5-HD had no effect on the rate of ATP synthesis, suggesting that mitochondrial K_{ATP} channels are not tonically active. These blockers also had no effect on recovery of function in normoxic hearts. In contrast, in hypoxic heart mitochondria, K_{ATP} channel blockers reduced the rates of ATP synthesis to rates similar to those observed in normoxic heart mitochondria. In hypoxic hearts, both K_{ATP} blockers significantly attenuated cardioprotection. These results corroborate our previous findings in isolated perfused hearts[1-3] and strongly suggest that enhanced activation of the mitochondrial K_{ATP} channel is an important component of the cardioprotective mechanisms involved in adaptation to hypoxic stress.

A second significant finding of these studies was the increased rate of ATP synthesis observed in mitochondria isolated from chronically hypoxic hearts. Moreover, there was no difference in myocardial ATP concentrations or in mitochondrial membrane potential in hypoxic versus normoxic hearts. One potential explanation for the apparent discrepancy between the inhibition of the rate of ATP synthesis observed in mitochondria isolated from normoxic immature rabbit hearts versus the enhanced rate of ATP synthesis after chronic hypoxia may be due to differences between acute versus chronic activation of the mitochondrial K_{ATP} channel. In the acute situation (ie, mitochondria isolated from normoxic hearts), activation of the mitochondrial K_{ATP} channel by K_{ATP} channel openers results in K^+ influx into mitochondria, mitochondrial depolarization, and a reduction in the driving force for ATP production measured in the present studies as a reduction in the rate of ATP synthesis. Our data further indicate that chronic hypoxia produces a tonic activation of the mitochondrial K_{ATP} channel. This is likely to result in adaptive changes in mitochondrial physiology. The observation that resting mitochondrial membrane potential did not differ between mitochondria isolated from normoxic or hypoxic hearts provides further evidence of an adaptive response to tonic activation of the mitochondrial K_{ATP} channel. Other studies have provided evidence that mitochondrial bioenergetics and metabolism are fundamentally altered by chronic hypoxia with changes reported in mitochondrial creatine kinase activity and in the ADP and O_2 dependence of mitochondrial respiration.[25,26] Our findings suggest that an alteration in mitochondrial K_{ATP} channel function may be another component in mitochondrial adaptation to hypoxia. Recent studies showing involvement of the mitochondrial K_{ATP} channel in adaptation to high-altitude hypoxia further support this interpretation.[27]

Taken together, our findings suggest that the cardioprotective effects of mitochondrial K_{ATP} channel activation may be linked to improved oxidative metabolism and mitochondrial bioenergetics. An important role of the mitochondrial K_{ATP} channel is to regulate mitochondrial volume, which in turn is thought to regulate electron transport and bioenergetics.[5,7-10] Opening of the mitochondrial K_{ATP} channel has been shown to shift the balance between K^+ uniport and K^+-H^+ antiport, resulting in transient net K^+ uptake and increased matrix volume.[5,8] Halestrap[7] has established that small increases in matrix volume stimulate electron transport and that activation of the mitochondrial K_{ATP} channel may trigger this response. Mitochondrial K_{ATP} channel activation may therefore be an essential component of a signal transduction pathway calling for increased ATP production to support increased work in the heart or possibly to compensate for decreased oxygen availability. Conversely, blockade of the mitochondrial K_{ATP} channel may interfere with the cellular or mitochondrial response to these signals. The reduction in the rate of ATP synthesis observed in mitochondria from hypoxic hearts treated with K_{ATP} channel blockers is consistent with this interpretation.

We have suggested that adaptation to chronic hypoxia represents a unique form of preconditioning, and we have recently supported this contention by showing that although immature normoxic hearts can be preconditioned, immature hypoxic hearts cannot be preconditioned.[3] Furthermore, we have shown that the mechanism of preconditioning in the immature normoxic heart is associated with K_{ATP} channel activation and is abolished by the mitochondrial K_{ATP} channel blocker 5-HD.[1,2] Although a direct link between mitochondrial K_{ATP} channel activation and myocardial protection remains to be established, several known consequences of mitochondrial K_{ATP} channel activation are likely to improve mitochondrial function after ischemia. Activation of the mitochondrial K_{ATP} channel results in K^+ influx into mitochondria, expansion of mitochondrial matrix volume, and a reduction of the inner mitochondrial membrane potential established by the proton pump.[5-10] Regulation of matrix volume is an essential element in the regulation of mitochondrial energy production, and matrix expansion secondary to mitochondrial K_{ATP} channel opening has been postulated to activate electron transport and stimulate mitochondrial metabolism.[7] Our findings of increased rates of ATP synthesis in mitochondria isolated from hypoxic hearts are consistent with this mechanism.

In summary, our data in conjunction with the studies of other investigators support a role for mitochondrial K_{ATP} channel activation and its impact on mitochondrial bioenergetics as an important factor in increased resistance to ischemia in hearts adapted to chronic hypoxia.

Acknowledgments

This work was supported by grants from National Heart, Lung, and Blood Institute (HL-08311 and HL-45048) and from the National Institute of Environmental Health Sciences (ES-06648). The authors wish to thank Patricia Holman for her excellent technical assistance.

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J Mol Cell Cardiol 33, 1041–1045 (2001)
doi:10.1006/jmcc.2001.1362, available online at http://www.idealibrary.com on IDEAL®

Exhibit G

Rapid Communication

Sarcolemmal and Mitochondrial K_{ATP} Channels Mediate Cardioprotection in Chronically Hypoxic Hearts

Xiangrong Kong[1], James S. Tweddell[2,4], Garrett J. Gross[3] and John E. Baker[1,3]

Divisions of [1]Pediatric Surgery and [2]Cardiothoracic Surgery, [3]Department of Pharmacology & Toxicology, Medical College of Wisconsin, Milwaukee, WI, USA and [4]Section of Cardiothoracic Surgery, Children's Hospital of Wisconsin, Milwaukee, WI 53226, USA

(Received 29 January 2001, accepted in revised form 9 February 2001, published electronically 9 March 2001)

X. Kong, J. S. Tweddell, G. J. Gross and J. E. Baker. Sarcolemmal and Mitochondrial K_{ATP} Channels Mediate Cardioprotection in Chronically Hypoxic Hearts. *Journal of Molecular and Cellular Cardiology* (2001) 33, 1041–1045. Hypoxia from birth increases the resistance of the isolated neonatal heart to ischemia. We determined if increased resistance to ischemia was due to activation of sarcolemmal or mitochondrial K_{ATP} channels. Rabbits ($n=8$/group) were raised from birth in a normoxic ($F_iO_2=0.21$) or hypoxic ($F_iO_2=0.12$) environment for 8–10 days and the heart perfused with Krebs–Henseleit bicarbonate buffer. A mitochondrial-selective K_{ATP} channel blocker 5-hydroxydecanoate (5-HD) (300 μmol/l) or a sarcolemmal-selective K_{ATP} channel blocker HMR 1098 (30 μmol/l) were added alone or in combination for 20 min prior to a global ischemic period of 30 min, followed by 35 min reperfusion. Recovery of ventricular developed pressure was higher in chronically hypoxic than normoxic hearts. 5-HD and HMR 1098 partially reduced the cardioprotective effect of chronic hypoxia, but had no effect in normoxic hearts. The combination of 5-HD and HMR 1098 abolished the cardioprotective effect of chronic hypoxia. We conclude that both sarcolemmal and mitochondrial K_{ATP} channels contribute to cardioprotection in the chronically hypoxic heart.

Key Words: 5-hydroxydecanoate; HMR 1098; K_{ATP} channel; Cardiovascular diseases; Hypoxia.

Introduction

Adaptation of the heart to chronic hypoxia from birth results in increased resistance to ischemia,[1] which is associated with activation of K_{ATP} channels.[2] Two subtypes of K_{ATP} channels exist: mitochondrial K_{ATP} channels, located in the inner mitochondrial membrane, and the surface K_{ATP} channels located in the sarcolemmal membrane. The cardioprotective effects of chronic hypoxia are abolished by glibenclamide,[3] a mitochondrial and sarcolemmal K_{ATP} channel blocker.[4] Thus, it is not known as to which channel mediates cardioprotection.

Selective openers and blockers of the mitochondrial and sarcolemmal K_{ATP} channels have been identified. The K_{ATP} channel opener diazoxide is 1000 times more selective for opening mitochondrial than sarcolemmal K_{ATP} channels.[5] The cardioprotective effect of diazoxide is abolished by the selective mitochondrial K_{ATP} channel blocker 5-hydroxydecanote (5-HD).[6] 5-HD also abolishes the cardioprotective effects of preconditioning in immature hearts.[7] The K_{ATP} channel blocker HMR 1883 and its sodium salt HMR 1098 are selective for the sarcolemmal K_{ATP} channel.[8] However, HMR 1098 does not block preconditioning[9] which suggests that the sarcolemmal K_{ATP} channel does not contribute to this form of cardioprotection.

The right ventricle is more resistant to ischemia in both normoxic and chronically hypoxic hearts.[3] However, the relative roles of mitochondrial and

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0022–2828/01/051041+05 $35.00/0

sarcolemmal K_{ATP} channels in mediating resistance to ischemia in the right ventricle are unknown. Thus, our objectives were to determine the contribution of the mitochondrial and sarcolemmal K_{ATP} channels to cardioprotection in left and right ventricle afforded by adaptation of hearts to chronic hypoxia.

Materials and Methods

Creation of hypoxia from birth

Rabbits were raised from birth to 8–10 days of age in a hypoxic ($F_IO_2 = 0.12$) or normoxic ($F_IO_2 = 0.21$) environment as described previously.[7]

Perfusion sequence

We performed the following experiments using eight groups ($n = 8$/group) to determine whether the mitochondrial or sarcolemmal K_{ATP} channels contribute to cardioprotection in chronically hypoxic hearts. The eight groups were as follows: group 1, normoxic, no intervention; group 2, normoxic, treated with 5-HD alone; group 3, normoxic, treated with HMR 1098 alone; group 4, normoxic, treated with 5-HD plus HMR 1098; group 5, chronically hypoxic, no intervention; group 6, chronically hypoxic, treated with 5-HD alone; group 7, chronically hypoxic, treated with HMR 1098 alone; group 8, chronically hypoxic, treated with 5-HD plus HMR 1098. Immediately after aortic cannulation, hearts were perfused in the Langendorff mode at a constant perfusion pressure of 42 mmHg[1] with balloons placed in left and right ventricles. Biventricular function and coronary flow rate were recorded under steady-state conditions.[5] 5-HD (300 μmol/l) or HMR 1098 (30 μmol/l) were added alone or in combination for 20 min prior to a global ischemic period of 30 min, followed by 35 min of reperfusion.

Recovery of developed pressure was expressed as a percentage of its pre-drug, pre-ischemic value. Results are expressed as the mean ± S.D. Statistical analysis was performed by use of repeated measures ANOVA with the Greenhouse–Geisser adjustment used to correct for the inflated risk of a Type I error.[3] If significant, the Mann–Whitney test was used as a second step to identify which groups were significantly different. After ANOVA the data were analysed for differences related to multiple comparisons.[3] Significance was set at $P < 0.05$.

Results

To determine the optimal concentration for 5-HD and HMR 1098 for use in the cardioprotection studies, we performed concentration-response studies for each drug (5-HD: 0–450 μmol/l, HMR 1098: 0–45 μmol/l) in chronically hypoxic hearts. In chronically hypoxic hearts both 5-HD and HMR 1098 exhibited a "U"-shaped response profile for recovery of left ventricular developed pressure and drug concentration. The optimal concentrations for reducing the cardioprotective effect of chronic hypoxia with 5-HD and HMR 1098 was 300 μmol/l and 30 μmol/l, respectively. In normoxic hearts 300 μmol/l 5-HD and 30 μmol/l HMR 1098 did not affect recovery of left ventricular developed pressure compared with drug free controls. These concentrations of 5-HD and HMR 1098 are able to block current through the mitochondrial and sarcolemmal K_{ATP} channels, respectively.[8]

Pre-ischemic function

Cardiac function and the effect of K_{ATP} channel blockers on aerobic function prior to ischemia were determined in immature normoxic and chronically hypoxic hearts (Table 1). 5-HD (300 μmol/l) did not affect heart rate, coronary flow or developed pressure in left or right ventricle in normoxic hearts. However, in chronically hypoxic hearts 5-HD depressed heart rate slightly without affecting coronary flow or developed pressure in either ventricle. HMR 1098 (30 μmol/l) did not affect heart rate, coronary flow or developed pressure in left or right ventricle in normoxic hearts. However, in chronically hypoxic hearts, HMR 1098 increased left but not right ventricular developed pressure and did not affect heart rate or coronary flow. The combination of 5-HD (300 μmol/l) plus HMR 1098 (30 μmol/l) had no effect on heart rate, coronary flow or left and right ventricular developed pressure in either normoxic or chronically hypoxic hearts.

Post-ischemic function

To determine the effect of chronic hypoxia on resistance to myocardial ischemia, recovery of post-ischemic function was examined in normoxic and hypoxic hearts not subjected to drug intervention. Recovery of left ventricular developed pressure following ischemia was greater in chronically hypoxic hearts (68 ± 4%) compared with normoxic hearts (44 ± 5%) (Fig. 1). Recovery of developed pressure

Table 1 Hemodynamic values for each group

Groups	Pre-drug				Post-drug				Reperfusion (35 min)			
	Heart rate (beats/min)	Coronary flow rate (ml/min)	Left ventricle developed pressure (mmHg)	Right ventricle developed pressure (mmHg)	Heart rate (beats/min)	Coronary flow rate (ml/min)	Left ventricle developed pressure (mmHg)	Right ventricle developed pressure (mmHg)	Heart rate (beats/min)	Coronary flow rate (ml/min)	Left ventricle developed pressure (mmHg)	Right ventricle developed pressure (mmHg)
1. Normoxic, no intervention	225±28	6±2	102±7	33±6	—	—	—	—	210±28	5±1	45±4†	23±6†
2. Normoxic+ 5-HD (300 μmol/l)	240±16	6±2	97±10	33±6	225±23	6±2	95±12	32±6	225±28	6±2	40±7†	24±4†
3. Normoxic+ HMR 1098 (30 μmol/l)	240±16	7±2	99±7	37±6	236±19	6±2	98±10	36±5	221±22	5±1	42±7†	23±3†
4. Normoxic+ 5-HD plus HMR 1098	248±14	6±2	101±5	35±4	248±14	6±2	107±7	36±3	236±19	6±2	45±6†	22±3†
5. Hypoxic, no intervention	221±16	9±2‡	100±8	53±3‡	—	—	—	—	210±23	8±1	67±4†‡	43±5†‡
6. Hypoxic+ 5-HD (300 μmol/l)	236±11	10±2	102±9	51±10	210±23*	9±2	103±11	51±11	210±23	7±2	53±5†	34±6†
7. Hypoxic+ HMR 1098 (30 μmol/l)	233±21	10±2	101±7	50±6	229±28	10±2	109±6*	54±7	218±35	7±2	55±4†	34±5†
8. Hypoxic+ 5-HD plus HMR 1098	244±11	11±2	103±6	50±5	244±11	11±3	109±7	55±6	229±28	8±3	45±5†	31±7†

Values are means±S.D., from 8 hearts /group. *=$P<0.05$, pre-drug *v* post-drug; †=$P=0.05$, pre-drug *v* reperfusion; ‡=$P<0.05$, normoxic *v* hypoxic.

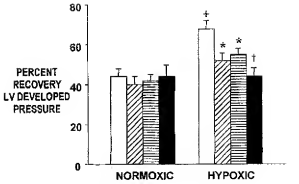

Figure 1 Recovery of left ventricular developed pressure following 20 min treatment with 5-HD alone (300 μmol/l), HMR 1098 (30 μmol/l) alone and 5-HD (300 μmol/l) combined with HMR 1098 (30 μmol/l) prior to 30 min global ischemia and 35 min reperfusion. (□), Control; (▨), 5-HD alone; (▤), HMR 1098 alone; (■), 5-HD combined with HMR 1098. Data are means ± S.D. ($n=8$ hearts/group). +, $P<0.05$, normoxic *v* hypoxic; *, $P<0.05$, drug alone *v* drug-free control; †, $P<0.05$, drug alone *v* drugs combined.

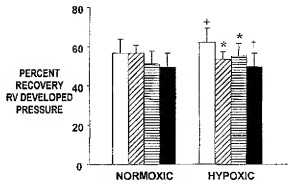

Figure 2 Recovery of right ventricular developed pressure following 20 min treatment with 5-HD alone (300 μmol/l), HMR 1098 (30 μmol/l) alone and 5-HD (300 μmol/l) combined with HMR 1098 (30 μmol/l) prior to 30 min global ischemia and 35 min reperfusion. (□), Control; (▨), 5-HD alone; (▤), HMR 1098 alone; (■), 5-HD combined with HMR 1098. Data are means ± S.D. ($n=8$ hearts/group). +, $P<0.05$, normoxic *v* hypoxic; *, $P<0.05$, drug alone *v* drug-free control; †, drugs combined *v* drug-free control.

in the right ventricle was greater in chronically hypoxic hearts (78 ± 8%) compared with normoxic hearts (71 ± 10%) (Fig. 2).

To determine the effect of blockade of mitochondrial and sarcolemmal K_{ATP} channels upon resistance to myocardial ischemia, recovery of postischemic function was measured in normoxic and chronically hypoxic hearts treated with 5-HD and HMR 1098 either alone or in combination prior to ischemia. 5-HD and HMR 1098 alone partially reduced recovery of left ventricular developed pressure in chronically hypoxic hearts to 52 ± 4% and 55 ± 3%, respectively, but had no effect in normoxic hearts (Fig. 1). However, the combination of 5-HD and HMR 1098 completely abolished the cardioprotective effect of chronic hypoxia (68 ± 4% to 44 ± 5%) but had no effect in normoxic hearts (Fig. 1). 5-HD and HMR 1098 alone completely abolished the cardioprotective effects of chronic hypoxia in the right ventricle (Fig. 2). The combination of 5-HD and HMR 1098 in chronically hypoxic hearts further depressed recovery of developed pressure in right ventricle to 62 ± 9% (Fig. 2).

Discussion

Previously we showed that chronic hypoxia from birth increased resistance of isolated hearts to ischemia, and that the cardioprotective effect of hypoxia was abolished by glibenclamide, a non-selective K_{ATP} channel blocker. However, the identity of the K_{ATP} channel subtype associated with increased resistance to ischemia remained unknown. In this report, we show that both mitochondrial and sarcolemmal K_{ATP} channels contribute to the cardioprotective effects of adaptation to chronic hypoxia from birth. The mitochondrial and sarcolemmal K_{ATP} channels did not contribute to cardioprotection in normoxic hearts. Simultaneous inhibition of both sarcolemmal and mitochondrial K_{ATP} channels completely abolished the cardioprotective effects of chronic hypoxia.

Our study is the first to demonstrate that cardioprotection induced by adaptation to chronic hypoxia involves activation of both the sarcolemmal and mitochondrial K_{ATP} channel. In contrast, cardioprotection induced by ischemic preconditioning involves the mitochondrial but not the sarcolemmal K_{ATP} channel.[9] Similarly, cardioprotection induced by opioids can also be abolished with 5-hydroxydecanoate but not HMR 1098.[10] These studies on cardioprotection induced by ischemic preconditioning and opioids were performed on unstressed normoxic hearts and these hearts may respond differently than those exposed to chronic hypoxia.

Most studies of cardioprotection have investigated resistance to ischemia in the left ventricle. The use of the isolated heart model allows simultaneous measurement of resistance to ischemia in both left and right ventricle and permits comparisons to be made. We showed that the right ventricle was more resistant to ischemia than the left ventricle in both normoxic and chronically

hypoxic hearts. The isolated heart model avoids the systemic effects of K_{ATP} channel openers or blockers. There is very little information available on the role of the K_{ATP} channel in mediating resistance to ischemia in the right ventricle. 5-HD and HMR 1098 were able to abolish the cardioprotective effects of chronic hypoxia in right ventricle indicating that mitochondrial and sarcolemmal K_{ATP} channels mediate resistance to ischemia in the chronically hypoxic right ventricle.

Cardioprotection of the myocardium can be induced by several ways including ischemic preconditioning[11] and chronic hypoxia.[1] However, distinct differences are present in the mechanisms underlying cardioprotection by ischemic preconditioning and adaptation to chronic hypoxia. In late preconditioning, nitric oxide generated from the NOS2 isoform protects the heart against sustained ischemia.[11] However, our studies with chronic hypoxia show nitric oxide generated from the NOS3 isoform is responsible for protecting the heart against ischemia.[12] Preconditioning is mediated by activation of the mitochondrial K_{ATP} channel.[9] Our study shows both sarcolemmal and mitochondrial K_{ATP} channels mediate cardioprotection in chronically hypoxic hearts. Thus, the operative mechanisms by which adaptation to chronic hypoxia and late preconditioning protect the heart against ischemia are separate.

We conclude the sarcolemmal and mitochondrial K_{ATP} channels contribute to cardioprotection in the chronically hypoxic heart. Further investigations are needed to clarify the mechanisms by which K_{ATP} channels become active during adaptation to chronic hypoxia and produce an increased resistance to myocardial ischemia.

Acknowledgements

This work was supported in part by grants HL-54075 and HL-08311 from the National Institutes of Health and the Children's Hospital of Wisconsin Foundation.

The authors gratefully acknowledge the secretarial assistance of Mary Lynne Koenig.

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Exhibit H

Chronic Hypoxia Increases Endothelial Nitric Oxide Synthase Generation of Nitric Oxide by Increasing Heat Shock Protein 90 Association and Serine Phosphorylation

Yang Shi, John E. Baker, Chenyang Zhang, James S. Tweddell, Jidong Su, Kirkwood A. Pritchard, Jr

Abstract—Chronic hypoxia increases endothelial nitric oxide synthase (eNOS) production of nitric oxide (·NO) and cardioprotection in neonatal rabbit hearts. However, the mechanism by which this occurs remains unclear. Recent studies suggest that heat shock protein 90 (hsp90) alters eNOS function. In the present study, we examined the role of hsp90 in eNOS-dependent cardioprotection in neonatal rabbit hearts. Chronic hypoxia increased recovery of postischemic left ventricular developed pressure (LVDP). Geldanamycin (GA), which inhibits hsp90 and increases oxidative stress, decreased functional recovery in normoxic and hypoxic hearts. To determine if a loss in ·NO, afforded by GA, decreased recovery, GA-treated hearts were perfused with *S*-nitrosoglutathione (GSNO) as a source of ·NO. GSNO increased recovery of postischemic LVDP in GA-treated normoxic and hypoxic hearts to baseline levels. Although chronic hypoxia decreased phosphorylated eNOS (S1177) levels by ≈4- to 5-fold and total Akt and phosphorylated Akt by 4- and 5-fold, it also increased hsp90 association with eNOS by more than 3-fold. Using hydroethidine (HEt), a fluorescent probe for superoxide, we found that hypoxic hearts contained less ethidine (Et) staining than normoxic hearts. Normoxic hearts generated 3 times more superoxide by an N^{ω}-nitro-L-arginine methyl ester (L-NAME)–inhibitable mechanism than hypoxic hearts. Taken together, these data indicate that the association of hsp90 with eNOS is important for increasing ·NO production and limiting eNOS-dependent superoxide anion generation. Such changes in eNOS function appear to play a critical role in protecting the myocardium against ischemic injury. (*Circ Res*. 2002;91:300-306.)

Key Words: chronic hypoxia ■ endothelial NOS ■ heat shock protein 90 ■ superoxide anion ■ nitric oxide

Nitric oxide plays an important role in protecting the heart against ischemic injury. *S*-nitrosoglutathione (GSNO), a nitric oxide (·NO) donor, improves functional recovery after ischemia, which is associated with increased cGMP.[1] Chronic hypoxia from birth in a neonatal rabbit model increases recovery of postischemic left ventricular developed pressure (LVDP) compared with recovery in normoxic hearts.[2] It is important to note that nitric oxide synthase (NOS) inhibitors, N^{ω}-nitro-L-arginine methyl ester (L-NAME) and N^{G}-methyl-L-arginine (L-NMA), decrease functional recovery of postischemic LVDP in hypoxic hearts after ischemia but do not decrease recovery in normoxic hearts.[2,3] These findings suggest that chronic hypoxia may alter the function of endothelial nitric oxide synthase (eNOS), the most abundant NOS isozyme in the rabbit heart, to increase cardioprotection.[2]

An increase in the association of heat shock protein 90 (hsp90) with eNOS increases production and activity of ·NO in response to growth factor stimulation.[4] Disruption of this protein-protein interaction decreases ·NO and blocks vasodilation in response to agonists.[4–7] Geldanamycin (GA), which inhibits conformational changes in hsp90[8] and increases oxidative stress by redox cycling,[9] has been shown to decrease ·NO and increase L-NAME–inhibitable superoxide generation in endothelial cells.[6] The role of hsp90 in modulating eNOS function in the heart has not been determined.

In the present study, we examine the role of hsp90 in modulating functional recovery of isolated hearts subjected to global ischemia. Using Western blot analysis, we determined how much hsp90 is associated with eNOS and the extent to which the enzyme is activated based on phosphorylation of eNOS at serine 1177.[10] The levels of superoxide from eNOS in the heart were assessed using NOS inhibitors and hydroethidine (HEt), an oxidant-sensitive fluorescent probe. Al-

Original received January 29, 2002; resubmission received June 7, 2002; revised resubmission received July 19, 2002; accepted July 22, 2002.

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Presented in part at the 74th Scientific Sessions of the American Heart Association, Anaheim, Calif, November 11–14, 2001, and published in abstract form (*Circulation*. 2001;104[suppl II]:II-263).

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DOI: 10.1161/01.RES.0000031799.12850.1E

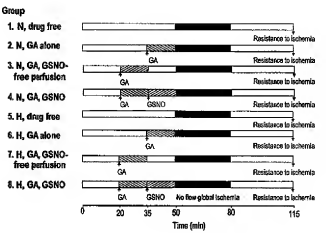

Figure 1. Experimental protocol used to study geldanamycin and GSNO in resistance of normoxic and chronically hypoxic hearts to ischemia. N indicates normoxia; GA, geldanamycin (18 μmol/L); GSNO, S-nitrosoglutathione (10 μmol/L); and H, chronic hypoxia. Open boxes represent aerobic perfusion; hatched boxes, perfusion with drug; and filled boxes, global ischemia.

though ·NO may play an important role in protection, the results of the present study suggest that one of the mechanisms by which hsp90 may protect the heart is by limiting superoxide generation from eNOS.

Materials and Methods

Animals

Animals used in this study received humane care in compliance with the Guide for the Care and Use of Laboratory Animals, by the National Research Council.

Creation of Hypoxia From Birth

Neonatal New Zealand White rabbits were obtained from New Franken Research Rabbits (New Franken, Wis) and were conditioned in normoxic and hypoxic environments as previously described.[2] Details of conditions are presented in an expanded Materials and Methods section, which can be found in the online data supplement available at http://www.circresaha.org.

Perfusion Studies

The protocol for perfusing isolated hearts with GA and subsequent ischemia is described in Figure 1. The protocol for perfusing isolated hearts with HEt and eNOS inhibitors is described in Figure 2. The

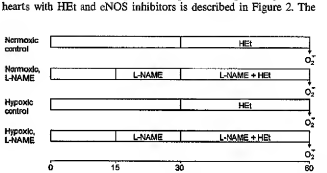

Figure 2. Experimental protocol used to determine the effects of chronic hypoxia on reactive oxygen species generation in the myocardium of hearts. Isolated hearts from normoxic and hypoxic rabbits were perfused with hydroethidine (HEt, 10 μmol/L) and/or L-NAME (400 μmol/L) at the times indicated.

hearts were perfused at 39°C in the Langendorff mode[11] at a perfusion pressure equivalent to 45 mm Hg.[12] The heart and perfusion fluids were immersed in nongassed physiological saline solution within temperature-controlled chambers to maintain the myocardium at 39°C, which is normothermic for rabbit. The standard perfusate was modified Krebs-Henseleit bicarbonate buffer[2]: (in mmol/L) NaCl 118.5; $NaHCO_3$ 25.0; KCl 4.8; $MgSO_4$ 0.6; H_2O 1.2; KH_2PO_4 1.2 (pH 7.4 when gassed with 95% O_2/5% CO_2) in which the calcium content was reduced to 1.8. Glucose (11.1 mmol/L) was added to the perfusate. Before use, all perfusion fluids were filtered through cellulose acetate membranes with pore size 5.0 μm to remove particulate matter.

Assessment of Ventricular Function

Left ventricular function was monitored continuously throughout each experiment as previously described.[13]

Tissue Sample Preparation

Hearts from normoxic and chronically hypoxic neonatal rabbits were isolated and perfused with aerobic bicarbonate buffer for 30 minutes at constant pressure. The free wall of the left ventricle was excised and immediately freeze-clamped between stainless steel tongs precooled with liquid nitrogen. Frozen myocardial tissue samples were powdered in a precooled stainless steel mortar and pestle. The powdered tissue was transferred to a dounce homogenizer with a Teflon pestle and homogenized in modified RIPA buffer (20 mmol/L Tris-HCl, pH 7.4, 2.5 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 100 mmol/L NaCl, 10 mmol/L NaF, 1 mmol/L Na_3VO_4, 1 mmol/L Pefabloc, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A) on ice for 50 strokes. Nuclei and cellular debris were removed by centrifugation (14000g×10 min). The supernatant was transferred to a cold microcentrifuge tube and protein concentrations determined by BCA protein assay (Pierce).

Immunoprecipitation and Western Analysis

Immunoprecipitation and Western analysis protocols were similar to the protocols in a previous report.[2,6] Experimental details for the protocols are provided in the online data supplement.

Detection of Superoxide Anion Generation in Isolated Hearts

The protocol for perfusion of hearts with HEt (10 μmol/L) and eNOS inhibitors, L-NAME (200 and 400 μmol/L), is shown in Figure 2. At the end of the perfusion, hearts were frozen in OCT 4583 and sectioned. Ten micron frozen sections were cut and thaw-mounted on slides. A coverslip was applied to the sections on the slides and images were obtained with a Nikon E600 microscope equipped with epifluorescence (Ex 488 nm, Em 610 nm) and a digital camera. The fluorescent intensity of nuclei in 40 cells from each animal was measured, corrected for background fluorescence in nonnuclear regions using MetaMorph software, and expressed as mean±SD arbitrary units of fluorescence.

Results

Effects of Geldanamycin and GSNO on Functional Recovery

Chronic hypoxia increased postischemic LVDP compared with that obtained in normoxic hearts ($P<0.01$, n=8). Geldanamycin decreased functional recovery of LVDP in normoxic and chronically hypoxic hearts by approximately the same degree ($P<0.01$, n=7 to 9 per group) (Figure 3). GSNO restored functional recovery in GA-treated normoxic and hypoxic hearts treated with GA to levels that were indistinguishable from initial baseline values. To control for the possibility that GSNO-dependent increases in recovery of postischemic LVDP in the GA-treated hearts were due to

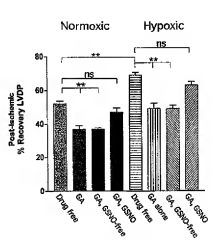

Figure 3. Effects of GA on functional recovery of postischemic LVDP. This bar graph shows LVDP in isolated perfused hearts from normoxic and chronically hypoxic neonatal rabbits. Hearts were perfused with bicarbonate buffer containing buffer alone, buffer containing GA (18 μmol/L), and buffer containing GSNO (10 μmol/L). Protocol pictured in Figure 1 was used to examine the effects of no-flow, global ischemia on functional recovery of LVDP. These data show that GA significantly decreases recovery of postischemic LVDP in both normoxic and hypoxic hearts and that recovery of postischemic LVDP to initial baseline levels can be restored by perfusion with GSNO (**$P<0.01$, n=7 to 9 per experimental test group).

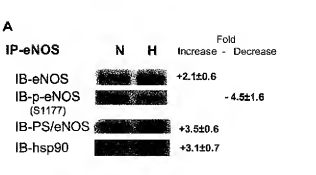

Figure 4. Effects of chronic hypoxia on the activation state of eNOS. A, This composite Western shows that chronic hypoxia in neonatal rabbit hearts increases eNOS protein, decreases phospho-eNOS on eNOS, increases immunodetectable levels of phosphoserine on eNOS and increases association of hsp90 on eNOS compared with eNOS in normoxic hearts. B, Western analysis for hsp90 content in homogenates of normoxic and chronically hypoxic hearts. H indicates hypoxic hearts; N, normoxic hearts; IP, immunoprecipitation; and IB, immunoblot.

perfusion alone, a third group was perfused for the same period of time as the GSNO group with GSNO-free bicarbonate buffer. Perfusion with bicarbonate buffer alone, after perfusion with GA, did not affect recovery of LVDP. The observation that GSNO increased LVDP to baseline levels for both normoxic and hypoxic hearts perfused with GA suggests that regardless of the mechanism by which GA increases susceptibility to ischemia, ·NO from GSNO is sufficient to restore LVDP to baseline values. These data are consistent with the fact that GA shifts the balance of ·NO and superoxide from ·NO toward superoxide anion.[6] These data confirm that shifting the balance of ·NO and superoxide toward superoxide increases susceptibility to ischemic injury and that restoring ·NO increases resistance to ischemia as proposed earlier.[2,14]

Effects of Chronic Hypoxia on the Activation State of eNOS

Previous studies showed that chronic hypoxia increased eNOS activity but not message levels.[2] In the present study, we find by Western analysis that chronic hypoxia increased eNOS levels in heart homogenates by 2.1±0.6-fold ($P<0.05$, n=6) (Figure 4A, first panel). As phosphorylation of eNOS at S1177 indicates the degree of electron flow through eNOS, we next measured phospho-eNOS (S1177) using a site-specific antibody.[6,10,15] The second panel of Figure 4A shows that chronic hypoxia decreased eNOS phosphorylation (S1177) compared with normoxic hearts (−4.5±1.6-fold, $P<0.05$, n=3). At first glance, these data seem to suggest that eNOS in hypoxic hearts might produce less ·NO than eNOS in normoxic hearts, which does not agree with previous findings.[2]

Because hsp90 increases ·NO generation from eNOS[10,15] and decreases superoxide from neuronal NOS (nNOS),[16,17] we next determined the extent to which hsp90 was associated with eNOS in normoxic and chronically hypoxic hearts. Chronic hypoxia increased the association of hsp90 with eNOS compared with normoxic hearts more than 3-fold (3.1±0.7-fold, $P<0.02$, n=6) (Figure 4A, fourth panel). These data demonstrate how important hsp90 is to coupling eNOS activity to L-arginine metabolism for the efficient generation of ·NO.[4,6] Although phospho-eNOS (S1177) may be important for increasing electron flow through the enzyme, increasing the association of hsp90 with eNOS appears to be sufficient to allow chronically hypoxic hearts to generate ≈2 times more ·NO than normoxic hearts.[2] To determine if the increase in association of hsp90 with eNOS is due to a change in hsp90 content, Western analysis of hsp90 in total heart homogenates was performed. Figure 4B shows that chronic hypoxia does not appreciably change the total content of hsp90 in the heart. Taken together, these data support the notion that the association of hsp90 plays an important role in helping eNOS generate ·NO, which protects against ischemic injury.

When the phosphorylation state of eNOS was examined with a general anti-phosphoserine antibody, we found that chronic hypoxia increased immunodetectable levels of phosphoserine on eNOS nearly 3- to 4-fold compared with that found in normoxic hearts ($P<0.05$, n=3; Figure 4A, third panel). As a first step in determining which site(s) on eNOS in rabbits could account for the increase in phosphoserine, we measured by Western analysis phospho-eNOS levels at S116 and T495 that have been reported to mediate eNOS function in other species.[18–20] Unfortunately, the commercially available antibodies did not detect bands of phosphorylation on eNOS from rabbits as they did for eNOS from bovine endothelial cells (Figure 5). The reasons for such differences in detection are unclear at this time but may be because the

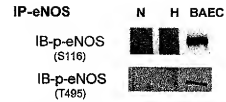

Figure 5. Western analysis for phosphorylation of eNOS. This composite Western of eNOS phosphorylation shows that site-specific antibodies against eNOS at S116 (human) and T495 (human) do not detect bands of phosphorylation on eNOS immunoprecipitated from normoxic and hypoxic rabbit hearts as it does for eNOS immunoprecipitated from cultured bovine aortic endothelial cells. H indicates hypoxic hearts; N, normoxic hearts; and BAEC, bovine aortic endothelial cells.

antibodies were raised in rabbits and/or because the antibodies were against phosphorylation sites in human eNOS, whose amino acid sequence may be different from the sequence for rabbit eNOS.

Effects of Chronic Hypoxia on Akt/Protein Kinase B

Within the signaling cascade for regulation of eNOS, Akt/protein kinase B is located immediately upstream.[15,21,22] On the basis of the data shown in Figure 4A (second panel), we predicted that chronic hypoxia may have altered signaling events leading to decreased phosphorylation of eNOS at S1177. Western analysis of Akt and phospho-Akt in lysates of heart homogenates revealed that chronic hypoxia dramatically decreased total Akt and phospho-Akt in hearts by 4- and 5-fold, respectively (Figure 6A). Because hsp90 did not change with chronic hypoxia, we performed Westerns for hsp90 and phospho-Akt on the same blot to control for loading. Figure 6B confirms findings in Figure 4B that hypoxia has little effect on hsp90 levels and shows that hypoxia seems to specifically decrease phospho-Akt levels, not induce generalized decreases in protein expression. These findings are consistent with the observation that chronic hypoxia decreased phosphorylation of eNOS at S1177. Furthermore, these data suggest that phosphorylation of other residues may regulate eNOS activity. However, using site-

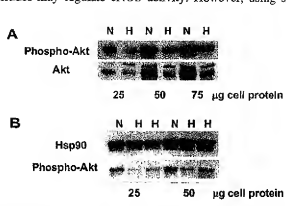

Figure 6. Effects of chronic hypoxia on Akt. A, This Western shows that chronic hypoxia decreases total Akt and phospho-Akt in neonatal rabbit hearts compared with levels in normoxic hearts. B, Western analysis for total hsp90 and phospho-Akt in normoxic and chronic hypoxic hearts. These blots show that chronic hypoxia had no effect on total hsp90 content but dramatically decreased phospho-Akt levels. H indicates hypoxic hearts; N, normoxic hearts.

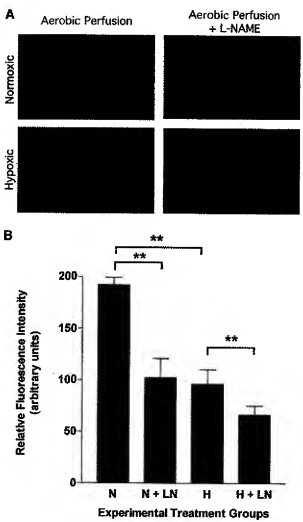

Figure 7. Effects of chronic hypoxia on Et staining in isolated perfused hearts: an index of superoxide anion generation. A, These images show the Et staining in the nuclei of normoxic and hypoxic hearts in the presence and absence of L-NAME. B, This bar graph shows the mean fluorescent intensity of Et staining in the nuclei of the myocardium in normoxic hearts and chronically hypoxic hearts after correction for nonnuclear fluorescence. These data reveal that in normoxic hearts, eNOS generates nearly 3 times more reactive oxygen products that increase Et staining than it does in hypoxic hearts. **$P<0.01$.

specific antibodies against phospho-eNOS (S116) and phospho-eNOS (T495) (human), we were unable to detect similar site-specific phosphorylation of rabbit eNOS, although phosphorylation of bovine eNOS at these sites was clearly evident (Figure 5).

Effects of Chronic Hypoxia on Uncoupled eNOS Activity

Based on the fact that phospho-eNOS (S1177) is a highly conserved site that directly correlates with electron flow through the enzyme,[10] that phosphoserine levels on eNOS have been shown to correlate directly with ·NO generation,[23] and that increased levels of hsp90 association limit superoxide anion generation from NOS,[6,16,17,24–27] we hypothesize that eNOS in chronically hypoxic hearts might be coupled

more efficiently to L-arginine metabolism than eNOS from normoxic hearts. To test this notion, we measured superoxide-dependent conversion of HEt to Et based on a previous report[25] in perfused normoxic and hypoxic hearts. Figure 7A shows that Et staining in normoxic hearts is significantly greater than staining in chronically hypoxic hearts. When the hearts were perfused with L-NAME (which blocks ·NO and superoxide anion production by eNOS[6]) Et staining was reduced to levels seen in hypoxic control hearts. L-NAME also reduced Et staining in chronically hypoxic hearts, albeit to a much smaller extent. When normoxic hearts were perfused with L-NMA, which inhibits ·NO but not superoxide generation from eNOS,[28] Et fluorescence increased markedly (data not shown). These reciprocal differences in the effects of the NOS inhibitors on Et staining in isolated perfused hearts are consistent with the fact that L-NAME blocks superoxide anion from eNOS, whereas L-NMA does not.[28] Image analysis and calculation of relative fluorescent intensities reveals that isolated perfused normoxic hearts generate nearly 3 times more superoxide by an L-NAME–inhibitable mechanism than chronically hypoxic hearts (Figure 7B). A marked increase in eNOS-dependent Et staining in normoxic hearts is consistent with the finding that phospho-eNOS (S1177) is high in normoxic hearts and with the finding that less hsp90 is associated with eNOS in normoxic hearts compared with hypoxic hearts. It is interesting to note that the low levels of L-NAME–inhibitable Et staining in the hypoxic hearts inversely correlated with an increase in general phosphoserine levels on eNOS (Figure 4A, third panel). These findings are consistent with our previous report that chronic hypoxia in neonatal rabbits maximally increases ·NO activity.[2]

Discussion

In this study, we show that geldanamycin (GA) decreases functional recovery of normoxic hearts and inhibits the beneficial effects of chronic hypoxia. Furthermore, we show that the deleterious effects of GA can be reversed by administration of ·NO. As chronic hypoxia increases resistance to ischemia by an L-NAME–inhibitable mechanism,[2,14] our findings suggest that hsp90 and an unidentified phosphoserine site on rabbit eNOS, likely different than S1177, act in concert to increase ·NO production and activity, as suggested in work by others.[4,23] These data suggest that the beneficial effects of chronic hypoxia are more closely related to how much hsp90 associates with eNOS than the magnitude of phosphorylation of eNOS at S1177 alone.[6] The observations that normoxic hearts contain nearly 5 times more phospho-eNOS (S1177) and generate 3 times more eNOS-dependent superoxide, however, are consistent with the fact that phosphorylation of eNOS at S1177 increases electron flow through the enzyme.[10] The relative changes in Et staining in these studies were seen predominantly in the myocytes, consistent with the observations that myocytes representing the majority of heart mass exhibit a diffuse pattern of staining for eNOS that colocalizes with caveolin-3 only at the sarcolemma and t-tubules.[29] On the basis of these observations, we conclude that that hsp90 plays an important role in increasing coupled eNOS activity, which not only increases ·NO production but also preserves ·NO biological activity.[6,16,17,27] Finally, our studies provide new insight into the cellular mechanisms by which adaptation to chronic hypoxia enhances coupled eNOS activity to increase cardioprotection.

Basic science studies using a variety of animal models clearly indicate ·NO plays a central role in cardioprotection. Ischemic preconditioning in rat,[30] canine,[31] and rabbit[32] protects hearts against ischemic reperfusion injury by increasing iNOS. Chronic hypoxia in the rat increases resistance to ischemia in isolated hearts.[33] Chronic hypoxia from birth in rabbits also confers resistance to ischemia.[12,13] Subsequent studies revealed that resistance was due to increased endogenous ·NO production and activity[2,3] and that eNOS, the most abundant transcript for the NOS isozyme family, was unaltered by chronic hypoxia.[3] Such findings indicated that adaptation to chronic hypoxia increases eNOS activity, but not necessarily eNOS mRNA expression to increase resistance to ischemia.[3]

Although the primary purpose of the study was to determine the mechanisms by which chronic hypoxia enhances eNOS activity to increase cardioprotection, a few words about how GA decreases cardioprotection are in order. GA is a well-recognized inhibitor of hsp90.[8] It also contains a semiquinone structure and is thus capable of redox cycling.[9] Accordingly, GA may inhibit functional recovery of isolated hearts by two mechanisms: decreasing ·NO generation via altering hsp90 interactions with eNOS[6] or decreasing ·NO activity via reaction with superoxide.[9] In additional studies, we found that GA decreased nitrite production by isolated hearts by more than half (1.69 ± 0.68 versus 0.77 ± 0.14 nmol/g per mL; $P<0.05$, n=6). In the studies shown in Figure 3, we see that GSNO restores functional recovery of GA-treated hearts to essentially baseline levels. If GA inhibited recovery solely by generating superoxide, then a decrease in nitrite production should not have occurred. If superoxide generated via redox cycling played a major role in decreasing cardioprotection, then GSNO should not have restored recovery of GA-treated hearts to baseline values.

Lucigenin and adriamycin are two well-recognized redox cycling agents that generate superoxide by interacting directly with the reductase domain of eNOS.[34,35] It is important to note that L-NAME does not block superoxide from eNOS when these agents are present.[34,35] The reason is that L-NAME is a substrate analogue inhibitor that only blocks eNOS activity at the arginine oxygenase domain, not the reductase domain.[34,35] With this information in mind, we perfused normoxic and hypoxic hearts with GA and HEt and then analyzed sections for relative levels of Et staining. We found that GA increased Et staining by $45 \pm 5.7\%$ (n=3) in normoxic hearts and $85 \pm 14\%$ (n=3) in hypoxic hearts, which L-NAME blocked as it did earlier.

On the basis that L-NAME is domain specific with respect to inhibiting eNOS-dependent superoxide generation, we conclude that GA increases superoxide anion generation, in a large part, from the arginine oxygenase domain. These findings are consistent with our previous report showing that L-NAME blocked ≈50% of the increase in superoxide generation in A23187-stimulated, GA-treated endothelial cell cultures,[6] reports showing that hsp90 increases eNOS gener-

ation of ·NO,[4,5,36,37] and the report showing that hypoxic hearts contain higher levels of eNOS activity and ·NO biological activity than normoxic hearts.[2] Taken together, these data and reports indicate that although GA can redox cycle to generate superoxide, its ability to inhibit hsp90 plays a major role in the mechanisms by which it decreases cardioprotection in isolated hearts.

To determine how chronic hypoxic increases eNOS activity, we examined the activation state of eNOS. Antibodies against sites of phosphorylation on human eNOS were obtained from commercial sources and used to examine the phosphorylation state of rabbit eNOS. On the basis that chronic hypoxia increases eNOS activity nearly 2-fold,[2] we expected to see a corresponding increase in phospho-eNOS (S1177) levels. Instead, the levels of phospho-eNOS at S1177 were decreased in chronically hypoxic hearts compared with normoxic controls. Further analysis using antibodies to the other phosphorylation sites on rabbit eNOS were unsuccessful, in that clear bands were not detected in samples from rabbits although bands could easily be detected in samples from bovine endothelial cells. The reason for this is unclear at this time. Sequence differences among species or the fact that the antibodies were raised in rabbits are possible explanations. As the antibodies were designed to be site-specific for human sequences, small differences in the amino acid sequence in rabbit eNOS may have been sufficient to prevent detection.

The association of hsp90 with eNOS is a universal mechanism among species for increasing ·NO generation. To date, this protein interaction has been observed in human, rodent, murine, canine, bovine, and ovine endothelial cells and cardiovascular tissues. The importance of this interaction to endothelial biology was recently confirmed by studies showing that hsp90 increased the efficiency of Akt-dependent phosphorylation of eNOS and that specific domains of hsp90 were responsible for delivering and directing Akt to S1179 on bovine eNOS.[38] In light of this information, the lower levels of Akt in homogenates of chronically hypoxic hearts provide a plausible explanation for the low levels of phospho-eNOS (S1177) on eNOS in chronically hypoxic hearts but not the more than 2-fold increase in eNOS activity we reported previously.[2] If one accepts that fact that the association of hsp90 increases eNOS generation of ·NO, then our findings suggest that hsp90 may be more important for increasing eNOS production of ·NO, as well as preserving the biological activity of ·NO, than increasing phospho-eNOS (S1177) levels alone. To determine if the increase in phospho-eNOS (S1177) observed in normoxic hearts still correlated with increased eNOS activity, superoxide, the product of uncoupled eNOS activity was measured. We found that eNOS-dependent Et staining was 3 times greater in normoxic hearts than in hypoxic hearts. Such data also support the idea that phospho-eNOS (S1177) directly correlates with electron flux through eNOS.[10] In the case of the normoxic hearts, however, this increased electron flux was weakly coupled to L-arginine metabolism, resulting in superoxide rather than ·NO generation. In contrast, an increase in general phosphoserine levels on eNOS in hypoxic hearts relative to those in normoxic hearts suggests that other sites of phosphorylation on eNOS also might influence enzyme function and, ultimately, cardioprotection. Future studies aimed at obtaining the full sequence for eNOS will be required to delineate mechanisms by which hsp90 interacts with eNOS in this species.

The possibility that direct protein interactions between hsp90 and eNOS preserves coupled enzyme activity is supported by recent findings by Song et al.[16,17] Using purified recombinant nNOS and hsp90 and spin-trapping with electron spin resonance to quantify ·NO production, Xia and associates[16,17] showed that activation of nNOS in the presence of hsp90 increased ·NO generation. In subsequent studies, they found that hsp90 also inhibited superoxide from nNOS and that this effect was more pronounced at lower L-arginine concentrations than at higher concentrations when hsp90 was present.[27] Another mechanism by which hsp90 might modulate eNOS function is by protecting sites of phosphorylation of eNOS. Using Western analysis, Granger and associates[23] found that VEGF increased phosphoserine residues on eNOS by a protein kinase C (PKC)–dependent mechanism that directly correlated with increased ·NO production and activity. This finding is consistent with those of Ping et al[39] using PKCε-GST-fusion proteins to demonstrate direct interactions between PKCε and eNOS. In the present study, using immunoprecipitation of eNOS and Western analysis, we find that chronic hypoxia markedly increased phosphoserine residues on eNOS even though phospho-eNOS (S1177) decreased. The decrease in phospho-eNOS (S1177) is supported by a marked reduction in total Akt and phospho-Akt, an immediate upstream kinase,[15,21,22] in hypoxic hearts. Our finding that chronic hypoxia increased phosphoserine residues on eNOS is consistent with reports that an increase in phosphoserine increases eNOS activity.[40–42]

These observations reveal how important it is for hsp90 to associate with eNOS when phospho-eNOS (S1177) levels are increased. Failure to increase hsp90 interactions with eNOS results in an inefficient coupling of enzyme activity to L-arginine metabolism and in an increase in eNOS-dependent superoxide generation. Our findings show that chronic hypoxia from birth increases cardioprotection of isolated hearts by increasing the association of hsp90 with eNOS. This critical protein interaction helps to couple eNOS activity to L-arginine metabolism and to limit superoxide anion generation. Such changes in radical species generation by eNOS increase ·NO production and help preserve ·NO activity in the heart, which increases resistance to ischemic reperfusion injury.

Acknowledgments

This work was supported in part by grants AHA 0020453Z (to Y.S.), HL54075 (to J.E.B), and HL61414 (to K.A.P.).

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Exhibit I

ERYTHROPOIETIN, NITRIC OXIDE SYNTHASE AND RESISTANCE TO MYOCARDIAL ISCHEMIA

Rabbits adapted to chronic hypoxia exhibit increased resistance to myocardial ischemia, resulting from increased nitric oxide production from endothelial nitric oxide synthase (1). However, the sensor responsible for detecting hypoxia resulting in increased nitric oxide production is unknown. The adequacy of renal tissue oxygenation at Epo-producing sites regulates Epo production (2), but a more potent extrarenal oxygen sensor may exist (3). L-NAME partially blocks increase in plasma levels of Epo in mice following exposure to hypoxia, thus implicating nitric oxide in oxygen sensing and Epo production (4). Epo directly stimulates atrial natriuretic peptide secretion from adult rat atria but not cultured myocyte (5). These data suggest Epo may play a role in adaptation of hearts to chronic hypoxia and resistance to ischemia by a NOS related mechanism.

Hypothesis 1: Chronic hypoxia results in increased Epo production that subsequently controls nitric oxide production from NOS.

1. Measure Epo receptors in normoxic and hypoxic hearts.
   Availability of antibody to Epo

Hypothesis 2: Epo increases nitric oxide production from NOS3.

2. Treat normoxic rabbits acutely with Epo, is there an increase in nitric oxide production resulting in cardioprotection.

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John E. Baker, Ph.D.
September 11, 2001

Exhibit J

Date: May 9, 2002

MCW Research Foundation
Discovery Record and Report

1. Brief descriptive title: Cardioprotection by Erythropoietin

2. Full name of discoverer(s), home address(es), and position(s):

   a. John E. Baker, Ph.D., 2131 N. 72 St., Wauwatosa, WI 53213 Professor

   b. Yang Shi, Ph.D., 2116 N. 115 St., Wauwatosa, WI 53226 Post doctoral fellow

   c.

3. Results to be achieved by the practice of this discovery:

   Improved resistance of the heart to ischemia.

4. Brief description of the discovery: (Attach additional pages of description if necessary).

   See attachment

5. Chronology of conception and reduction to practice:

   a. Date of earliest conception: [redacted]

   b. Date of disclosure (orally or in writing) to other persons and names of such persons: [redacted]

   c. First written record pertinent to discovery: [redacted]

   d. Date and result of first test of the discovery: 12/19/01

6. Source, number and size of grant(s) used to support the research relating to this discovery:

   Departmental funding and NIH HL54075 $[redacted]

7. Date and place of publication or anticipated publication: (Attach copy of publication if available.)

   Autumn 2002

8. List any published information on known practices in the field of the discovery which is pertinent:

Witness: Mary Lynne Koenig

Discoverer: JE Baker

Name: John E. Baker, Ph.D. Date May 9, 2002

Yang Shi

Name: Yang Shi, Ph.D. Date May 9, 2002

Name: _____ Date

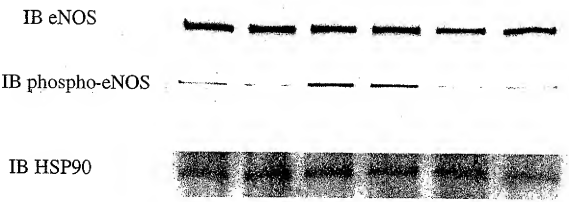
Exhibit J
continued

4. Brief description of the discovery

Erythropoietin is a key blood glycoprotein that initiates and regulates red blood cell production. Erythropoietin is approved by the FDA for human use in the treatment of anemia. We determined if erythropoietin can increase the resistance of the heart to ischemia. Hearts from New Zealand White rabbits were perfused with erythropoietin (0.5 – 10.0 U/ml) for 15 min prior to a global ischemic insult of 30 min followed by 35 min reperfusion. Erythropoietin exhibited a dose-dependent cardioprotective effect with optimal cardioprotection observed at 1.0 U erythropoietin/ml. Cardioprotection was manifest by a highly significant increase in recovery of pre-ischemic left ventricular developed pressure from 48±3% to 75±4%. We believe this is the first demonstration of cardioprotection by erythropoietin.

11-2-01

IP eNOS



		C1	C2	EPO1	EPO2	VEGF1	VEGF2
Ratio:	phospho-eNOS/eNOS	1		6.1		0.6	

Exhibit J
continued

EPO 5units/ml treatment for 24 hrs

IP eNOS

IB  Phospho-eNOS

IB  Hsp90

C1 C2 EPO1 EPO2 VEGF1 VEGF2

Exhibit J
continued

Exhibit K

Activation of Protein Kinases in Chronically Hypoxic Infant Human and Rabbit Hearts

Role in Cardioprotection

Parvaneh Rafiee, PhD; Yang Shi, PhD; Xiangrong Kong, MD; Kirkwood A. Pritchard, Jr, PhD; James S. Tweddell, MD; S. Bert Litwin, MD; Kathleen Mussatto, RN; Robert D. Jaquiss, MD; Jidong Su, MD; John E. Baker, PhD

Background—Many infants who undergo heart surgery have a congenital cyanotic defect in which the heart is chronically perfused with hypoxic blood. However, the signaling pathways by which infant hearts adapt to chronic hypoxia and resist subsequent surgical ischemia is unknown.

Method and Results—We determined the activation and translocation of protein kinase C (PKC) isoforms and mitogen activated protein kinases (MAP kinases) in 15 infants with cyanotic (Sao_2<85%) or acyanotic (Sao_2>95%) heart defects undergoing surgical repair and in 80 rabbits raised from birth in a hypoxic (Sao_2<85%) or normoxic (Sao_2>95%) environment. Tissues from infant human and rabbit hearts were processed for Western and in vitro kinase analysis. In human infants with cyanotic heart defects, PKCε, p38 MAP kinase, and JUN kinase but not p42/44 MAP kinase were activated and translocated from the cytosolic to the particulate fraction compared with acyanotic heart defects. In rabbit infants there was a parallel response for PKCε, p38 MAP kinase, and JUN kinase similar to humans. In infant rabbit hearts inhibition of PKCε with chelerythrine, p38 MAP kinase, with SB203580 and JUN kinase with curcumin abolished the cardioprotective effects of chronic hypoxia but had no effects on normoxic hearts.

Conclusions—Infant human and rabbit hearts adapt to chronic hypoxia through activation of PKCε, p38 MAP kinase, and JUN kinase signal transduction pathways. These pathways may be responsible for cardioprotection in the chronically hypoxic infant rabbit heart. ***(Circulation. 2002;106:239-245.)***

Key Words: hypoxia ■ ischemia ■ proteins ■ heart defects, congenital ■ heart diseases

Many infants who undergo cardiac surgery have a congenital cyanotic defect in which the heart is chronically perfused with hypoxic blood. However, the signaling pathways by which infant hearts adapt to chronic hypoxia and resist subsequent surgical ischemia is unknown.

By elucidating the impact that prolonged periods of hypoxia exerted on resistance to subsequent ischemia, we should be able to improve cardioprotection in infants with congenital heart defects.

Protein kinase C (PKC) family members are important mediators of hypoxia. In cardiomyocytes, PKCα and PKCε translocate from soluble to particulate fractions of the cell in response to the stress of chronic hypoxia.[1] The mitogen-activated protein kinases (MAP kinases) are ubiquitous proteins activated by diverse stimuli and appear to mediate cellular responses including proliferation, differentiation, and adaptation to stress.[2] Three major MAP kinase families have been characterized, including the extracellular signal–regulated kinases (ERK or p42/44 MAPK), the c-Jun NH_2-terminal kinases (JUN kinase), and the p38 MAP kinases (p38 MAPKs).[2] ERKs are mainly involved in mediating anabolic processes such as cell division, growth, and differentiation; the JUN kinases and the p38 MAPK are generally associated with cellular response to diverse stresses. The clinical relevance of protein kinases in adult humans was recently demonstrated by an increased activity of JUN kinase and p38 MAPK in heart failure secondary to ischemic heart disease[3] and during cardiopulmonary bypass.[4] However, the role of PKC and MAPKs in the mechanisms by which infant hearts adapt to chronic hypoxia and resist subsequent surgical ischemia are unknown.

To examine the role of these signaling pathways in adaptation to chronic hypoxia we identified and characterized PKC and MAPKs in hearts from human infants with cyanotic (Sao_2<85%) or acyanotic (Sao_2>95%) heart defects and in hearts from infant rabbits raised from birth in a hypoxic (Sao_2<85%) or normoxic (Sao_2>95%) environment. We then determined the contribution of PKC and MAPKs to

Received March 21, 2002; revision received April 26, 2002; accepted May 1, 2002.

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DOI: 10.1161/01.CIR.0000022018.68965.6D

Patient Characteristics

	Cyanotic (n=7)	Acyanotic (n=6)
Age, mo		
Mean	4.8±0.9	5.8±1.2
Range	1 wk to 9 mo	1 to 10 mo
Body weight, kg	3.9±0.4	5.2±0.5*
Sex, male/female	4/3	4/4
Pathology		
CAVC	0	2
VSD	0	2
TOF	2	0
AS	0	1
DORV	1	0
PAVC	0	3
HLHS	4	0
Hemoglobin, g/dL	15.6±0.6	12.5±1.3*
Blood O_2 saturation, %	73±5	98±1*

CAVC indicates complete atrioventricular canal; VSD, ventricular septal defect; TOF, tetralogy of Fallot; AVSD, atrioventricular septal defect; AS, aortic stenosis; DORV, double-outlet right ventricle with transposition of the great arteries; ASD, atrial septal defect; PAVC, partial atrioventricular canal; and HLHS, hypoplastic left heart syndrome.

*$P<0.05$, cyanotic vs acyanotic.

cardioprotection in chronically hypoxic and normoxic infant rabbit hearts. Our studies reveal that many of the protein kinase signaling mechanisms activated by chronic hypoxia in infant rabbits are identical to those activated by cyanotic heart defects in human infants. Once activated, we show that protein kinases confer cardioprotection in the chronically hypoxic infant rabbit heart.

Methods

Humans

The use of human tissue in this study was approved by the Human Research and Review Committee at Children's Hospital of Wisconsin and the Medical College of Wisconsin. Fifteen infants undergoing elective open heart surgery for congenital heart defects were prospectively recruited for this study. To determine whether protein kinases are activated by chronic hypoxia, the patients were divided into cyanotic and acyanotic groups according to blood oxygen saturation (acyanotic, $SaO_2>95\%$; cyanotic, $SaO_2<85\%$). All cyanotic patients were stable, with $SaO_2<85\%$ for 24 hours before surgery. There were no emergency operations performed on acutely hypoxic patients. Right atrial tissue (≈200 mg) from infants with congenital acyanotic and cyanotic heart defects was harvested at the time of surgical repair. The tissue was immediately frozen in liquid nitrogen and processed for Western analysis as described previously.[5] Preoperative characteristics are summarized in the Table.

Rabbits

Animals used in this study received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" formulated by the National Research Council, 1996. Infant rabbits were maintained for 10 days in a hypoxic ($SaO_2<85\%$) or normoxic ($SaO_2>95\%$) environment as described previously.[6]

Isolated Heart Perfusion

Isolated rabbit hearts (n=8/group) perfused in a retrograde manner and instrumented as previously described.[6]

Effect of PKC and MAPK Inhibitors

Hearts from normoxic or chronically hypoxic rabbits were perfused in the Langendorff mode. Biventricular function and coronary flow were recorded under steady-state conditions.[6] Hearts were then perfused for 15 minutes with vehicle, chelerythrine (1 μmol/L), SB203580 (15 μmol/L), curcumin (10 μmol/L), or PD98059 (10 μmol/L) before 30 minutes of global normothermic (39°C) ischemia and 40 minutes of reperfusion. Recovery of developed pressure was expressed as a percentage of its predrug, preischemic value. Results are expressed as mean±SD.

To determine the effect of chelerythrine and SB203580 on protein kinases in chronically hypoxic and normoxic hearts, isolated hearts (n=4 to 7 per group) were aerobically perfused with these drugs for 15 minutes. The free wall of the left ventricle was then processed to obtain cytosolic and particulate fractions[7] for Western analysis, as described previously.[5]

SDS-PAGE and Western Blot Analysis

Equal concentrations of protein were analyzed by SDS-PAGE and Western blotting by using either isoform-specific antibodies for phospho-PKC detection or specific antibodies against phosphorylated and nonphosphorylated p38 MAPK, JNK, and p42/44 MAPK (Cell Signaling Technology). The blots were developed by ECL. Densitometry was performed on each sample and analyzed with the use of NIH image software. Phosphorylated Hsp27 and PKCε were detected with the use of specific antibodies from Upstate Biotechnology Inc. Total PKC activity was measured by a PKC kit from Amersham, according to the manufacturer's instructions.

Immunoprecipitation and In Vitro Kinase Assays

To determine MAPK activity, nonradioactive kinase assay kits were used (Cell Signaling). p38 MAPK activation in normoxic and hypoxic infant human hearts was determined by measurement of its catalytic activity with the use of the in-gel kinase assay using GST-MAPKAPK-2, rHsp27, and GST-ATF-2 as substrate according to the manufacturer's instructions.

Phosphorylation of Threonine 71 on ATF-2

Aliquots of nuclear and cytosolic fractions were subjected to Western analysis with the use of specific phospho-ATF-2 (Thr71) antibody or control anti-ATF-2 as described previously.[8] The purity of the fractions was confirmed with antibody markers specific for the cytosolic and nuclear compartments β-actin and histone deactylase-1, respectively, with separation confirmed by Western analysis.[9]

Statistical Analysis

Statistical analysis was performed by use of repeated measures ANOVA with the Greenhouse-Geisser adjustment used to correct for the inflated risk of a type I error.[6] If significant, the Mann-Whitney test was used as a second step to identify which groups were significantly different. After ANOVA the data were analyzed for differences related to multiple comparisons.[6] Significance was set at $P<0.05$.

Results

Adaptation to Chronic Hypoxia

PKC and MAPK in Human Heart

To determine the involvement of PKC and MAPKs in normoxic and hypoxic hearts, cytosolic and particulate fractions were examined by SDS-PAGE and Western analysis with the use of specific monoclonal and polyclonal antibodies. Our results indicate that in normoxic hearts, multiple PKC isoforms (α, β, γ, ε, δ, and ζ) are present in the cytosolic fractions. However, adaptation to chronic hypoxia results only in the translocation of PKCε from cytosolic fraction to the particulate fraction (Figure 1).

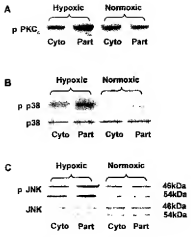

Figure 1. Chronic hypoxia in infant human heart results in phosphorylation and translocation of PKCε, p38 MAP kinase, and JUN kinase from cytosolic to particulate fraction. Cytosolic and particulate fractions were analyzed by Western blotting using phospho-specific antibodies against A, PKCε; B, p38 MAPK; and C, JNK. Nonphosphorylated antibodies were used to confirm equal loading of proteins for p38 MAPK and JNK. Cyto indicates cytosolic; Part, particulate.

Next, we sought to determine if the MAPK pathways play a role in adaptation to chronic hypoxia. We have shown that in normoxic hearts, phospho-p38 MAPK is present in both cytosolic and particulate fractions, but chronic hypoxia results in an increase of phospho-p38 MAPK in the particulate fraction (Figure 1). We also found that chronic hypoxia activates JUN kinase in human heart. Chronic hypoxia did not result in activation of phospho-p42/44 MAPK in human hearts. We confirmed that equal amounts of p38 and JNK proteins were analyzed by stripping and reprobing the same blots with control anti-p38 and anti-JNK antibodies (Figure 1).

We examined whether activation and translocation of PKCε, p38 MAPK, and JUN kinase was related to the variability in clinical presentation of the two groups of patients studied (Table). In all hearts adapted to chronic hypoxia, there was activation and translocation of protein kinases. In contrast, activation and translocation did not occur in any of the normoxic hearts. Thus, in all cases, the changes we observed in protein kinase activation and translocation were solely dependent on oxygen deprivation and not to the underlying clinical presentation responsible for the congenital defect.

p38 MAPK plays a protective role during adaptation to ischemic preconditioning by phosphorylating MAPKAPK-2, which in turn phosphorylates Hsp27.[10] Activation of this pathway is cardioprotective and overexpression of Hsp27 confers protection against ischemia in myocytes.[11] To determine if this pathway is present in human infants and activated by adaptation to chronic hypoxia, we probed normoxic and hypoxic hearts for changes in MAPKAPK-2 and Hsp27. Chronic hypoxia induced activation and translocation of both MAPKAPK-2 and Hsp27 from the cytosolic to the particulate fraction. Neither MAPKAPK-2 nor Hsp27 was activated in normoxic hearts (Figure 2).

p38 MAPK also transduces signals from the cytoplasm to the nucleus in response to cellular stress. ATF2 is a transcription factor phosphorylated by p38 MAPK.[8] To determine if this holds in hearts adapted to chronic hypoxia, phosphorylation of GST-ATF2 by p38 MAPK was determined in hearts from normoxic and chronically hypoxic infants. Our results demonstrate that phospho-p38 MAPK immunoprecipitates

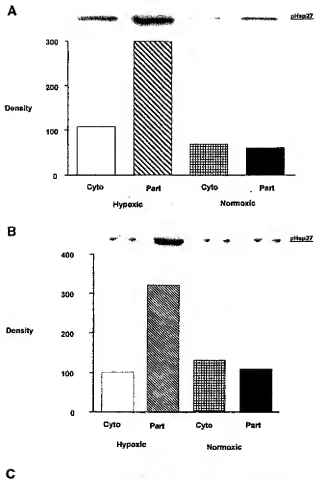

Figure 2. Chronic hypoxia in infant human heart activates MAPKAPK-2 and phosphorylates Hsp27 and ATF-2 (Thr 71). A, In vitro kinase assay shows phosphorylation of substrate Hsp27 by MAPKAPK-2 in particulate fraction. B, Chronic hypoxia-induced Hsp27 phosphorylation in particulate fraction. C, Chronic hypoxia results in ATF-2 (Thr 71) phosphorylation in nuclear fraction. Cyto indicates cytosolic; Part, particulate; and Nucl, nuclear.

from chronically hypoxic hearts result in phosphorylation of GST-ATF-2 in the particulate fraction (Figure 2).

ATF-2 Phosphorylation in Nuclear Fraction of Hypoxic Hearts

Transcriptional activity of ATF-2 can be stimulated by JNK and p38 MAPK. ATF-2 binds to both AP-1 and cAMP response element. Therefore, we examined whether chronic hypoxia phosphorylates and activates ATF-2. Our results show that adaptation to chronic hypoxia phosphorylates Thr71 of ATF-2 in the nuclear fraction (Figure 2), suggesting activation of this transcription factor. We confirmed that equal amounts of ATF-2 protein were analyzed by stripping and reprobing the same blots with control anti–ATF-2 antibody.

PKC and MAPK in Rabbit Heart

We found an identical pattern of activation for PKCε, and MAPKs in isolated perfused hearts from rabbits adapted to chronic hypoxia. Chronic hypoxia also induced activation of both MAPKAPK-2 and Hsp27 in the particulate fraction. This pattern of activation was also present in freshly excised hearts not subjected to perfusion before analysis. To determine the relative upstream/downstream positions of PKCε, p38 MAPK, and JUN kinase in the signal transduction pathway activated by chronic hypoxia, hearts were perfused with specific inhibitors of PKC and p38 MAPK, followed by Western blot analysis of the heart lysates. Perfusion of isolated rabbit hearts with chelerythrine, an inhibitor of PKC, reversed the translocation of PKCε, p38 MAPK, and JUN kinase in chronically hypoxic rabbits but had no effect in normoxic rabbits (Figure 3). Perfusion of hearts with SB203580, an inhibitor of p38 MAPK, also reverses the translocation of p38 MAPK but not PKCε or JUN kinase in chronically hypoxic hearts. SB203580 had no effect in normoxic rabbit hearts (Figure 4). These data suggest PKCε is an upstream kinase for activation of p38 MAPK and JUN kinase in chronically hypoxic rabbit hearts. SB20380 also prevented activation of ATF-2 by p38 MAPK in chronically hypoxic hearts. Our data shows that many of the protein kinase signaling mechanisms activated by chronic hypoxia in infant rabbit hearts are identical to those activated by cyanotic heart defects in infant human hearts.

We determined whether protein kinase activation in chronically hypoxic rabbit hearts is altered by subsequent perfusion with bicarbonate buffer. Excised hearts not subjected to subsequent perfusion and excised hearts subjected to 45 minutes of aerobic perfusion were freeze-clamped. Western analysis of PKCε and p38 MAPK revealed no differences in the extent of activation between the two groups. These data indicate the initial period of perfusion exerted no effect on protein kinase activation. To determine the ability of curcumin to specifically inhibit JNK rather than p38 MAPK normoxic hearts were perfused with anisomycin (20 μmol/L). Curcumin (10 μmol/L) completely blocked anisomycin-induced phosphorylation of JNK and minimally blocked phosphorylation of p38 MAPK. These data indicate curcumin selectively inhibits JNK with minimal effects on p38 MAPK (Figure 5).

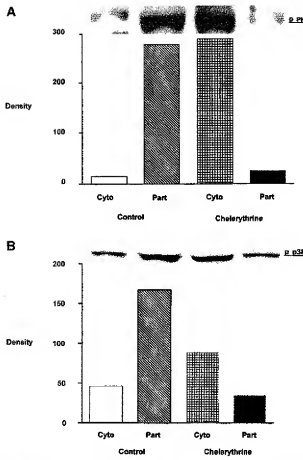

Figure 3. Effect of chelerythrine, a PKC inhibitor, on PKCε and p38 MAPK in chronically hypoxic rabbit heart. Cytosolic and particulate fractions were analyzed by Western blotting with specific antibodies against A, phospho-PKCε, and B, phospho-p38 MAPK. Chelerythrine significantly inhibited translocation of both PKCε and p38 MAPK from cytosolic to particulate fraction in hypoxic rabbit heart. Cyto indicates cytosolic; Part, particulate.

Parallel Response to Right Atria and Left Ventricle to Chronic Hypoxia

Human atrial but not ventricular tissues were readily obtainable for study. In contrast, rabbit ventricular and atrial tissue were both readily obtainable. However, we did not know if the adaptive response of left ventricle to chronic hypoxia parallels that of right atria. The degree of chronic hypoxia in the atria may not reflect that of the ventricle. We determined the impact of chronic hypoxia on PKCε and p38 MAPK activation and translocation in left ventricle and right atria from chronically hypoxic rabbits. Chronic hypoxia resulted in activation and translocation of PKCε and p38 MAPK in both left ventricle and right atria (Figure 6). These data demonstrate right atrial tissue responded to the same extent as left ventricle to chronic hypoxia. Thus, right atria are suitable to study chronic hypoxia-induced changes in protein kinase activation.

Resistance to Ischemia

Cardiac function and the effects of protein kinase inhibitors on aerobic function before ischemia were determined in infant normoxic and chronically hypoxic rabbit hearts. Coronary flow rate was 18% higher in hypoxic hearts than

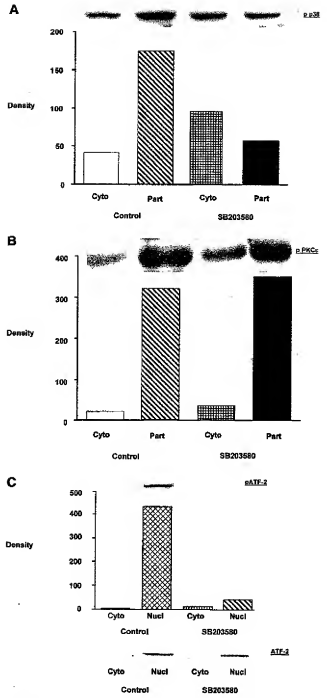

Figure 4. Effect of SB203580, a p38 MAPK inhibitor, on p38 MAPK, PKCε, and ATF-2 in chronically hypoxic rabbit heart. Cytosolic, particulate, and nuclear fractions were probed with specific antibodies against A, phospho-p38 MAPK; B, phospho-PKCε; and C, phosphorylated and nonphosphorylated ATF-2. SB203580 inhibits translocation of p38 MAPK from cytosolic to particulate fraction in hypoxic rabbit heart but did not inhibit translocation of PKCε. SB203580 inhibits phosphorylated but not nonphoshorylated ATF-2 in the nuclear fraction of hypoxic rabbit heart. Cyto indicates cytosolic; Part, particulate; and Nucl, nuclear.

normoxic controls as an adaptive response to increased oxygen delivery to the myocardium. Right ventricular developed pressure was higher in chronically hypoxic hearts than in normoxic hearts as a consequence of right ventricular

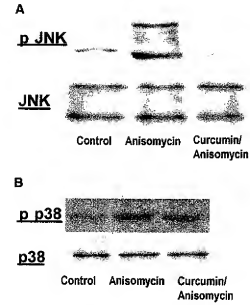

Figure 5. Effect of curcumin on JUN kinase and p38 MAPK in normoxic rabbit heart. Isolated hearts were perfused with anisomycin alone (20 μmol/L) for 15 minutes and then with anisomycin (20 μmol/L) plus curcumin (10 μmol/L) for 15 minutes. Cell lysates were probed with specific antibodies against A, JNK, and B, p38 MAPK. Anisomycin activated JNK and p38 MAPK. Curcumin completely blocked phospho-JNK activation and minimally blocked phospho-p38 MAPK activation. P indicates phosphorylated antibody.

hypertrophy. Chelerythrine (1 μmol/L), SB203580 (15 μmol/L), curcumin (10 μmol/L), and PD98059 (10 μmol/L) did not exert any affect on heart rate, coronary flow, or developed pressure in left or right ventricle in normoxic or chronically hypoxic hearts before ischemia. To determine the effect of chronic hypoxia on resistance to myocardial ischemia, recovery of postischemic function, was examined in infant normoxic and hypoxic hearts not subjected to drug intervention. Recovery of developed pressure in the left ventricle after ischemia was greater in chronically hypoxic hearts compared with normoxic controls (Figure 7). To determine the effect of inhibition of PKC, p38 MAPK, JUN kinase, and p42/44 MAPK on resistance to myocardial ischemia, recovery of postischemic function was measured in normoxic and hypoxic hearts perfused with chelerythrine, SB203580, curcumin, and PD98059 before ischemia. Neither chelerythrine, SB203580, curcumin, nor PD98059 affected resistance to ischemia in normoxic hearts. In contrast, chelerythrine, SB203580, and curcumin completely abolished the cardioprotective effects of chronic hypoxia. PD98059 did not affect recovery of postischemic function in chronically hypoxic hearts. Recovery of postischemic function in the right ventricle for all drugs paralleled the change observed in the left ventricle.

Discussion

Previously, we showed that chronic hypoxia in infant rabbits increases resistance of the heart to global ischemia.[6] However, the mechanisms by which hearts adapt to chronic hypoxia and resist subsequent ischemia remain unknown. In

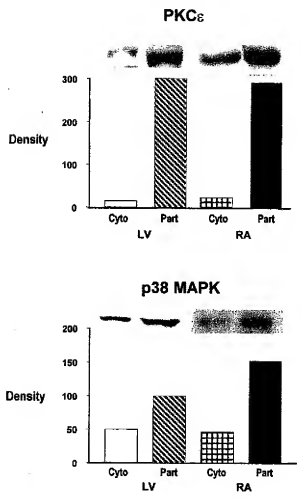

Figure 6. Parallel response of right atria and left ventricle to chronic hypoxia in infant rabbit. Chronic hypoxia resulted in activation of phospho-PKCε and phospho-p38 MAPK in both left ventricle and right atria. Cyto indicates cytosolic; Part, particulate; LV, left ventricle; and RA, right atrium.

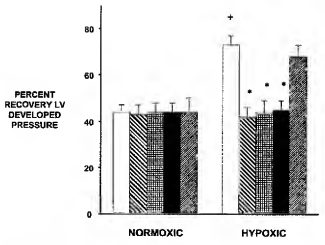

Figure 7. Recovery of left ventricular developed pressure in infant rabbit heart after 15 minutes of treatment with chelerythrine (1 μmol/L), SB203580 (15 μmol/L), curcumin (10 μmol/L), and PD98059 (10 μmol/L) before 30 minutes of global ischemia and 35 minutes of reperfusion. Control (□); chelerythrine (▨); SB203580 (▩); curcumin (▪); and PD98059 (▥). LV indicates left ventricle. Data are mean±SD (n=8 hearts/group). +$P<0.05$, normoxic vs hypoxic, *$P<0.05$, drugs vs control.

the present study, we have demonstrated that infant human and rabbit hearts adapt to chronic hypoxia through PKCε, p38 MAPK, and JUN kinase activation but not p42/44 MAPK. Our data also reveal that many of the protein signaling mechanisms activated by chronic hypoxia in infant rabbits are identical to those activated in infant humans. Activation of PKCε, p38 MAPK, and JUN kinase but not p42/44 MAPK mediates cardioprotection in chronically hypoxic infant rabbits.

Adaptation to Chronic Hypoxia

Chronically hypoxic human infant and rabbit hearts demonstrated activation of PKCε,which was evident by translocation of the PKCε isoform from the cytosolic to the particulate fraction. PKCε but not the α, β, δ, γ, and ζ isoforms of PKC were phosphorylated and translocated in hearts adapted to chronic hypoxia. PKCε is critical for cardiac myocyte protection by hypoxic preconditioning in a cell culture model.[12] Changes in specific PKC isoforms located in the myocardium have been reported, particularly in ischemic preconditioning, ischemia-reperfusion, heart failure caused by cardiomyopathy, and diabetes.[7,13–15] Our studies indicate activation of PKCε is an important adaptive response to chronic hypoxia.

Chronic hypoxia results in activation of p38 MAPK and JUN kinase but not p42/p44 MAPK in both human and rabbit hearts. Phosphorylation and activation of Hsp27 a substrate for p38 MAPK was present in chronically hypoxic infant hearts but not in normoxic hearts. We demonstrated that chronic hypoxia also caused phosphorylation of ATF-2, a substrate for p38 MAPK. We believe this is the first evidence of activation of protein kinase signaling pathways in infant human hearts in response to the stress of chronic hypoxia. In chronically hypoxic rabbit hearts, inhibition of PKCε by chelerythrine prevents the activation and the translocation of PKCε and p38 MAPK but not p42/44 MAPK. Inhibition of p38 MAPK by SB203580 did not inhibit PKCε translocation in chronically hypoxic hearts. Thus in chronically hypoxic rabbit hearts, PKCε appears upstream of the p38 MAPK pathway.

Adaptation to chronic hypoxia appears to stimulate phosphorylation of protein kinases to convert them from an inactive to an active state. Once activated, protein kinases translocate from the cytosolic to the particulate fraction, where their presence is associated with increased cardioprotection. Inhibition of activated PKCε, p38 MAPK, and JNK reverses this chronic hypoxia-induced translocation of protein kinases, resulting in the abolition of cardioprotection. To explain this novel observation, we propose adaptation to hypoxia maintains protein kinases in a chronically active state with activation maintained by a mechanism involving continuous shuttling of protein kinases between the cytosolic and particulate fractions. These events would in turn maintain activation of nuclear transcription factors resulting in altered expression of target genes that confer cardioprotection.

Resistance to Myocardial Ischemia

Perfusion of rabbit hearts before ischemia with inhibitors of PKCε, p38 MAPK, and JUN kinase alone abolished the cardioprotective

effects of chronic hypoxia but had no effect in normoxic hearts. Inhibition of p42/44 MAPK by PD98059 before ischemia had no effect on cardioprotection in normoxic and chronically hypoxic hearts, confirming our findings that p42/44 MAPK does not play a role in chronically hypoxic hearts.

Cardioprotection induced by adaptation to chronic hypoxia may involve changes in the actin cytoskeleton. Activation of p38 MAPK activates MAPKAP-2, which can in turn phosphorylate Hsp27,[16] an important regulator of actin dynamics that promotes polymerization of actin filaments, thus increasing the stability of the cytoskeleton.[17] Activation of p38 MAPK has been shown to prevent cytochalasin D–induced fragmentation of actin filaments, thus preserving cell viability.[17,18] Furthermore, overexpression of Hsp27 in isolated rat ventricular myocytes confers protection against simulated ischemia.[11] Because prolonged ischemia is known to cause cytoskeleton disruption, activation of the MAPKAPK-2/Hsp27 pathway and preservation of the actin filaments may explain some of the cardioprotective effects of adaptation to chronic hypoxia. In addition, phosphorylated Hsp27 interacts with Daxx, a mediator of Fas-induced apoptosis, preventing the interaction of Daxx with both Ask1 and Fas to block Daxx-mediated apoptosis.[19] Cardioprotection by adaptation to chronic hypoxia is also associated with activation of sarcolemmal and mitochondrial K_{ATP} channels.[20] PKC activates the sarcolemmal K_{ATP} channel by phosphorylation of the pore forming Kir6.2 subunit.[21] Thus, activation of PKC by chronic hypoxia may mediate cardioprotection by regulating K_{ATP} channel function.

The limitations of our study are that we could not identify the cell type in which PKCε and MAPKs are activated. In addition, resistance to ischemia in hearts from human infants at the time of surgical repair was not measured. The proposed role of PKC and MAPKs in the signal transduction pathway by which infant hearts adapt to chronic hypoxia and resist subsequent ischemia has been based on experiments with kinase inhibitors. This pharmacological approach is dependent on the relative specificity of the inhibitors. For example SB203580 inhibits p38α, β, and β2 but not γ and δ isoforms of p38 MAPK. SB203580 does not inhibit PKC and JNK. Chelerythrine inhibits all PKC isoforms and can activate MAPK pathways. Curcumin inhibits several kinases upstream of JNK and is an antioxidant. PD98059 is a potent and selective inhibitor of MEK, an upstream kinase of p42/44 MAPK.

We conclude infant human and rabbit hearts adapt to chronic hypoxia through activation of PKCε, p38 MAPK, and JUN kinase. It appears that these pathways are responsible for cardioprotection in the chronically hypoxic infant rabbit heart. Protection of the infant heart during surgical repair of congenital heart defects remains incomplete.[22] Exploitation of one or more of these protein kinase signaling pathways may afford increased cardioprotection to human infants undergoing repair of congenital heart defects.

Acknowledgments

This work was supported by grants HL-54075 (Dr Baker), HL-61414 (Dr Pritchard) from the National Heart, Lung, and Blood Institute, Ronald McDonald's Children's Charities (Dr Baker), and Children's Hospital of Wisconsin Foundation (Drs Tweddell and Baker).

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